Proceedings of the 7th Congress on Plant Protection

Доклады 7-ого Конгресса по защите растений

Plant Protection Society of Serbia
Общество по защите растений Сербии

International Organization for Biological Control
-East Palearctic Regional Section (IOBC-EPRS)
-West Palearctic Regional Section (IOBC-WPRS)

Международная организация по биологической борьбе
- Восточно палеарктическая региональная секция (МОББ-ВПРС)
- Западно палеарктическая региональная секция (МОББ-ЗПРС)

Editors/Редакторы

Dejan Marčić
Milka Glavendekić
Philippe Nicot

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„Integrated Plant Protection – a Knowledge-Based Step towards Sustainable Agriculture, Forestry and Landscape Architecture“

(November 24-28, 2014, Zlatibor, Serbia)

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„Интегрированная защита растений - научно обоснованный шаг к устойчивому развитию сельского хозяйства, лесоводства и пейзажной архитектуры“

(24-28 ноября 2014 года, Златибор, Сербия)

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on the occasion of the 60th anniversary of the PPSS
по поводу 60-летия ОЗРС

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Editors/Редакторы

Dejan Marčić

Institute of Pesticides and Environmental Protection
Banatska 31B, P.O.Box 163, 11080 Belgrade, Serbia

Milka Glavendekić

University of Belgrade, Faculty of Forestry
Kneza Višeslava 1, 11000 Belgrade, Serbia

Philippe Nicot

INRA, UR407 Pathologie végétale
F-84140 Montfavet, France

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PREFACE

The Plant Protection Society of Serbia (PPSS) and two regional sections of the International Organization for Biological and Integrated Control (IOBC-EPRS and IOBC-WPRS), on the occasion of the 60th anniversary of the PPSS organized VII Congress on Plant Protection with a motto: *"Integrated Plant Protection – a Knowledge-Based Step towards Sustainable Agriculture, Forestry and Landscape Architecture"* (November 24-28, 2014, Zlatibor, Serbia). The Congress enabled exchange of up-to-date scientific and technical information on plant protection in Agriculture, Forestry and Landscaping among researchers, teachers, experts in extension and public services and the business community, and promoted international cooperation. The Congress focused on basic knowledge and management practices established in plant protection, as well as on the development of alternative and innovative approaches. In addition, biological control as an important tool for the control of the harmful organisms with a minimal risk for ecosystems was discussed. A total of 209 contributions was presented - 8 keynote presentations, 28 oral presentations and 173 poster presentations - prepared by 467 authors from 26 countries. The Congress Proceedings comprise 65 contributions - 5 keynote presentations and 60 oral and poster presentations in six sessions, prepared by the authors from 18 countries (Algeria, Austria, Bosnia-Herzegovina, France, Georgia, Hungary, Italy, Kazakhstan, Montenegro, Poland, Russia, Rwanda, Serbia, Slovenia, Switzerland, Turkey, Uganda, USA). All contributions were reviewed by members of the Scientific Committee and other reviewers selected and invited by the editors of this publication.

Belgrade, November 2015 Editors

ПРЕДИСЛОВИЕ

Общество по защите растений Сербии (ОЗРС), Международная организация по биологической борьбе с вредными животными и растениями - Восточно палеарктическая региональная секция (МОББ-ВПРС) и Международная организациая по биологической борьбе и интегрированной системе защиты растений - Западно-палеарктическая региональная секция (МОББ-ЗПРС), по поводу 60-летия ОЗРС организировали VII Конгресс по защите растений, под девизом: *"Интегрированная защита растений - научно обоснованный шаг к устойчивому развитию сельского хозяйства, лесоводства и пейзажной архитектуры"* (24-28 ноября 2014 года, Златибор, Сербия). Цель Конгресса была обеспечение континуитета взаимообмена научно-техническими информациями, отвечающими современным требованиям защиты растений в сельском хозяйстве, лесоводстве и пейзажной архитектуре, которые представляют интерес для ученых, исследователей, преподавателей, экспертов-советников в области сельского хозяйства, лесоводства и пейзажной архитектуры, специалистов государственных и коммунальных служб, деловых кругов и средств массовой информации. Целью Конгресса является и продолжение содействия развитию и популяризации международного сотрудничества. Конгресс был концентрирован на основные знания и практический менаджмент в защите растений, а также на развитие алтернативних и новых подходов. Биологическая защита каторая представляет значительный способ для безопасной борьбы с вредними организмими была тоже рассмотривана. На конгрессе представлено 209 презентаций - 8 докладов по приглашению, 28 устных и 173 постер презентаций - которые подготовило 467 авторов из 26 стран. Сборник имеет 65 докладов - 5 докладов по приглашению и 60 устных и постер презентаций, распределенных в шести секциях. Авторы докладов приехали из 18 стран (Алжир, Австрия, Босния-Герцеговина, Франция, Грузия, Венгрия, Италия, Казахстан, Черногория, Польша, Россия, Руанда, Сербия, Словения, Швейцария, Турция, Уганда, США). Рецензенты всех опубликованных докладов в сборнике – члены Научного совета и другие рецензенты, выбранные редакторам этого издания.

Белград, Ноября 2015 Редакторы

Contents/Содержание

KEYNOTE PRESENTATIONS - ДОКЛАДЫ ПО ПРИГЛАШЕНИЮ

INTEGRATED PROTECTION OF FIELD CROPS, VEGETABLES AND STORED PRODUCTS

ИНТЕГРИРОВАННАЯ ЗАЩИТА ПОЛЕВЫХ И ОВОЩНЫХ РАСТЕНИЙ И ПРОДУКТОВ В СКЛАДСКИХ ПОМЕЩЕНИЯ

INTEGRATED PROTECTION OF FRUIT CROPS

ИНТЕГРИРОВАННАЯ ЗАЩИТА ФРУКТОВЫХ НАСАЖДЕНИЙ

BIOPESTICIDES AND BENEFICIAL ORGANISMS IN AGRICULTURAE

БИОПЕСТИЦИДЫ И ПОЛЕЗНЫЕ ОРГАНИЗМЫ В СЕЛЬСКОМ ХОЗЯЙСТВЕ

INTEGRATED PROTECTION IN FORESTRY AND LANDSCAPE ARCHITECTURE

ИНТЕГРИРОВАННАЯ ЗАЩИТА В ЛЕСНОМ ХОЗЯЙЦТВЕ И ПЕЙЗАЖНОЙ АРХИТЕКТУРЕ

HARMFUL ORGANISMS IN AGRICULTURE

ОРГАНИЗМЫ-ВРЕДИТЕЛИ Б СЕЛЬСКОМ ХОЗЯЙСТВЕ

TOXICOLOGY AND ECOTOXICOLOGY
ТОКСИКОЛОГИЯ И ЭКОТОКСИКОЛОГИЯ

KEYNOTE PRESENTATIONS

ДОКЛАДЫ ПО ПРИГЛАШЕНИЮ

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ДЕЯТЕЛЬНОСТЬ ЕОКЗР ПО ПРИМЕНЕНИЮ АГЕНТОВ БИОЛОГИЧЕСКОЙ БОРЬБЫ ПРОТИВ КАРАНТИННЫХ ВРЕДНЫХ ОРГАНИЗМОВ

Мартин Уорд[1] и Андрей Дорианович Орлинский[2]

1Секретариат Европейской и Средиземноморской организации по карантину и защите растений (ЕОКЗР) / European and Mediterranean Plant Protection Organization (EPPO) Secretariat; 21 Bd Richard Lenoir, Paris, 75011 France, mw@eppo.int.
2Секретариат Европейской и Средиземноморской организации по карантину и защите растений (ЕОКЗР) / European and Mediterranean Plant Protection Organization (EPPO) Secretariat; 21 Bd Richard Lenoir, Paris, 75011 France, Orlinski@eppo.int.

АБСТРАКТ

ЕОКЗР начала работы, связанные с агентами биологической борьбы, в 1997 году. Была создана «Группа экспертов ЕОКЗР по интродукции экзотических агентов биологической борьбы». С 1997 по 2002 год группа разработала три стандарта ЕОКЗР: PM 6/1 «Первый завоз экзотических агентов биологической борьбы для исследований в изолированных условиях», PM 6/2 «Импорт и выпуск в природу неаборигенных агентов биологической борьбы» и PM 6/3 «Перечень агентов биологической борьбы, широко применяемых в регионе ЕОКЗР». Последний содержит «Позитивный перечень» агентов биологической борьбы (АББ), которые являются аборигенными для региона ЕОКЗР или используются как минимум пятью странами ЕОКЗР в течение как минимум пяти лет без каких-либо отрицательных нецелевых последствий. Этот перечень является рекомендацией странам – членам ЕОКЗР использовать упрощённую процедуру интродукции и выпусков в природу тех АББ, которые в него включены. Группа экспертов приняла решение ограничить сферу деятельности беспозвоночными агентами биологической борьбы (БАББ), поскольку применение микро-АББ регулируется в рамках правил в отношении препаратов для защиты растений.

Группа возобновила свою работу в 2008 году под названием «Совместная группа экспертов ЕОКЗР и МОББ по агентам биологической борьбы», обновила «Положительный перечень» и продолжила обновлять его ежегодно, пересмотрела стандарт ЕОКЗР PM 6/2. Был разработан перечень информации, необходимой для отдельного БАББ, чтобы рассмотреть возможность его включения в «Положительный перечень».

Деятельность ЕОКЗР по биологической борьбе до сих пор фокусировалась на аспектах безопасности применения БАББ. Сейчас рассматривается возможность расширить спектр деятельности на работы по оценке эффективности БАББ, их применению в подавлении популяций вредных организмов для ограничения их распространения в новые зоны и для снижения их вредоносности до приемлемого уровня. Рассматривается вопрос разработки схемы оценки экологического риска для БАББ, которых предполагается интродуцировать в новые зоны. Такая схема должна оценивать потенциальные риски для окружающей среды в результате интродукции БАББ, а также положительное экологическое воздействие БАББ благодаря подавлению популяций вредных организмов и сокращения негативного воздействия на окружающую среду альтернативных методов защиты растений, применяемых при отсутствии БАББ.

Ключевые слова: Европейская и Средиземноморская организация по карантину и защите растений, беспозвоночный агент биологической борьбы, оценка экологического риска, карантинный вредный организм, группа экспертов.

ВВЕДЕНИЕ

Работы по карантину и защите растений в мире координируются Международной Конвенцией по карантину и защите растений (МККЗР) и десятью региональными организациями по карантину и защите растений, самой большой из которых является Европейская и Средиземноморская организация по карантину и защите растений (ЕОКЗР) (Арнитис, Орлинский, 2012). МККЗР является, с одной стороны, многосторонним договором о международном сотрудничестве в области карантина и защиты растений, под которым подписалась 181 договаривающаяся сторона (по состоянию на конец 2014 года), а с другой стороны – организацией со штаб-квартирой в Риме, разрабатывающей Международные стандарты по фитосанитарным мерам (МСФМ), главная задача которых – гармонизация подходов стран к мерам по предотвращению интродукции и распространению на их территориях вредных для растений организмов.

ЕОКЗР – межправительственная региональная организация, со штаб-квартирой в Париже, основанная в 1951 году, имеющая в своём составе (на конец 2014 года) 50 стран и обеспечивающая сотрудничество этих стран как в области фитосанитарных регламентаций (карантина растений), так и в области применения препаратов для защиты растений. Каждым из этих двух направлений руководит соответствующая рабочая группа. Рабочие группы, составленные из представителей стран ЕОКЗР, ежегодно принимают решения о приоритетных направлениях работ организации и рассматривают проекты стандартов ЕОКЗР. Решения рабочих групп ежегодно утверждаются Советом ЕОКЗР – главным административным органом организации.

Конкретную работу по направлениям, принятым рабочими группами и утверждённым Советом, проводят группы экспертов (ГЭ) по более узким направлениям. Так, Рабочая группа ЕОКЗР по фитосанитарным регламентациям руководит работой таких групп экспертов, как ГЭ по фитосанитарным мерам, ГЭ по карантинным лесным вредным организмам, ГЭ по развитию анализа фитосанитарного риска (АФР), ГЭ по энтомологии, ГЭ по диагностике и качеству лабораторий, ГЭ по инвазивным чужеродным растениям и т.п. Рабочая группа ЕОКЗР по препаратам для защиты растений руководит работой таких групп экспертов, как ГЭ по оценке эффективности фунгицидов и инсектицидов, ГЭ по общим стандартам по оценке эффективности препаратов, ГЭ по резистентности вредных организмов к препаратам для защиты растений и т.п. Группы экспертов разрабатывают рекомендации ЕОКЗР странам, в большинстве в виде стандартов, по различным вопросам в рамках своих компетенций. После апробации этих стандартов соответствующей рабочей группой и утверждения Советом эти стандарты публикуются в Бюллетене ЕОКЗР и на сайте организации в Интернете (www.eppo.int).

Организационными вопросами занимается Секретариат ЕОКЗР, который поддерживает сайт организации в Интернете, собирает и распространяет для стран фитосанитарную информацию, создаёт и поддерживает ряд баз данных и компьютерных программ (таких как программа CAPRA по проведению АФР; базы данных EPPT, – по таксономии и кодам для более чем 60000 организмов, и PQR, содержащая данные по биологии, распространению и растениям-хозяевам для более чем 1500 видов вредных для растений организмов), издаёт Бюллетень ЕОКЗР, организует более 30 международных встреч в год, включая совещания групп экспертов и рабочих групп ЕОКЗР, а также семинары, симпозиумы и конференции. Всю эту информацию и базы данных можно найти на сайте организации в Интернете.

ИСТОРИЯ ДЕЯТЕЛЬНОСТИ ЕОКЗР ПО БИОЛОГИЧЕСКОМУ МЕТОДУ ЗАЩИТЫ РАСТЕНИЙ

ЕОКЗР начала работы, связанные с агентами биологической борьбы, после семинара по безопасности и эффективности биологической борьбы в Европе (EPPO/CABI Workshop on Safety and Efficacy of Biological Control in Europe), организованного совместно ЕОКЗР и CABI в Стритли (Великобритания) в марте 1996 года (Streatley, GB, 1996-03-26/28). На этом семинаре были рассмотрены вопросы истории и развития биометода в ряде европейских стран (EPPO/CABI, 1997a; Orlinski, 1997). В соответствии с рекомендациями семинара (EPPO/CABI, 1997b) в 1997 году была создана «Группа экспертов ЕОКЗР по интродукции экзотических агентов биологической борьбы» («EPPO Panel on Introduction of Exotic Biological Control Agents») в рамках Рабочей группы ЕОКЗР по фитосанитарным регламентациям. В эту группу вошли как представители национальных организаций по карантину и защите растений (НОКЗР) стран ЕОКЗР, так и эксперты,

представляющие две секции (западно палеарктическую и восточно палеарктическую) Международная организация по биологической борьбе с вредными животными и растениями (МОББ). В 2000 году название группы было изменено на «Группу экспертов ЕОКЗР по агентам биологической борьбы» («EPPO Panel on Exotic Biological Control Agents»), в 2001 году – на «Группу экспертов ЕОКЗР по экзотическим агентам биологической борьбы» («EPPO Panel on Biological Control Agents»), а в 2002 году – на «Группу экспертов ЕОКЗР по безопасному применению биологической борьбы» («EPPO Panel on Safe Use of Biological Control»). Необходимо отметить, что Техническая группа экспертов по Глоссарию МККЗР приняла решение о некорректности использования термина «экзотический» применительно к организмам и целесообразности его замены терминами «неаборигенный» или «не местный». Это нашло отражение в «Глоссарии фитосанитарных терминов» МККЗР (IPPC, 2014) и в других международных стандартах по фитосанитарным мерам.

В течение первых лет работы (1997 – 2002 гг.) группа экспертов разработала три стандарта ЕОКЗР серии PM 6 «Безопасное применение биологической борьбы» («Safe use of biological control»): PM 6/1 «Первый завоз экзотических агентов биологической борьбы для исследований в изолированных условиях» («First import of exotic biological control agents for research under contained conditions»; EPPO, 1999), утверждённый в 1999 году, PM 6/2 «Импорт и выпуск в природу неаборигенных агентов биологической борьбы» («Import and release of non-indigenous biological control agents»; EPPO, 2001), впервые утверждённый в 2000 году, и PM 6/3 «Перечень агентов биологической борьбы, широко применяемых в регионе ЕОКЗР» («List of biological control agents widely used in the EPPO region»; EPPO, 2002), впервые утверждённый в 2001 году. Эти и другие стандарты ЕОКЗР можно найти в открытом доступе на сайте организации в Интернете. Последний из трёх перечисленных выше стандартов (PM 6/3) содержит так называемый «Позитивный перечень» агентов биологической борьбы (АББ), которые являются аборигенными для региона ЕОКЗР или используются как минимум пятью странами ЕОКЗР в течение как минимум пяти лет без наблюдения каких-либо отрицательных нецелевых последствий. Этот перечень является рекомендацией ЕОКЗР странам – членам организации использовать упрощённую процедуру интродукции и выпусков в природу тех АББ, которые в него включены, поскольку их безопасность считается доказанной. С самого начала своей работы группа экспертов приняла решение ограничить сферу своей деятельности беспозвоночными агентами биологической борьбы (БАББ), поскольку применение микро-АББ регулируется в рамках правил в отношении препаратов для защиты растений (как химических, так и нехимических).

С 2002 года группа экспертов не собиралась в течение нескольких лет, поскольку считалось, что она выполнила поставленные задачи. Такая ситуация продолжалась вплоть до 2008 года, когда Совет ЕОКЗР принял решение о необходимости ежегодного обновления «Положительного перечня» минуя сложную процедуру пересмотра самого стандарта PM 6/3, то есть под ответственность группы экспертов и без консультации со странами и одобрения Рабочей группой ЕОКЗР по фитосанитарным регламентациям. Поэтому группа экспертов возобновила свою работу под названием «Совместная группа экспертов ЕОКЗР и МОББ по агентам биологической борьбы», которое сохраняется до сегодняшнего дня. Группа обновила «Положительный перечень» и продолжила обновлять его ежегодно, а также значительно пересмотрела стандарт ЕОКЗР PM 6/2. Эта версия стандарта, PM 6/2(2), была утверждена Советом ЕОКЗР в 2010 году (EPPO, 2010) и, с небольшими поправками, PM 6/2(3) – в 2014 году (EPPO, 2014). Группой был разработан перечень информации, которая должна быть собрана о конкретном БАББ для рассмотрения решения о его включении в «Положительный перечень». Была разработана процедура по удалению из «Положительного перечня» тех видов БАББ, которые более не удовлетворяют разработанным для перечня критериям. В настоящее время стандарт PM 6/3 включает в себя три списка: (1) «Коммерчески применяемые БАББ», (2) «Успешно интродуцированные БАББ в рамках классического биометода» и (3) «БАББ, ранее рекомендованные ЕОКЗР». Первые два представляют собой «Положительный перечень», а третий включает виды, удалённые из него. В настоящее время в него выведены из «Положительного перечня» всего три вида: *Cales noacki*, *Harmonia axyridis* и *Lysiphlebus testaceipes*. При этом факт удаления какого-либо вида БАББ из «Положительного перечня» не означает, что страны ЕОКЗР не должны его применять, но им не рекомендуется использовать упрощённую процедуру для его интродукции и выпусков в природу. Последнюю версию стандарта PM 6/3(4) можно найти на сайте ЕОКЗР в Интернете

по адресу http://archives.eppo.int/EPPOStandards/biocontrol_web/bio_list.htm.

Группа экспертов изучила правила интродукции и выпусков в природу БАББ, а также применение «Положительного перечня», в разных странах ЕОКЗР. Как выяснилось, положение сильно различается между странами. Если в некоторых странах действуют очень жёсткие правила, регламентирующие интродукцию и выпуски в природу БАББ (например, в Швейцарии, Франции, Великобритании), то в ряде стран никаких правил в этом отношении вообще не существует. «Положительный перечень» в некоторых странах является одним из факторов, влияющих на принятие решения об интродукции БАББ, наряду с подробным досье и проведением оценки экологических рисков. В других странах присутствие организма в «Положительном перечне» служит определяющим фактором для разрешения интродукции. Наконец, есть страны, в которых отсутствие вида в «Положительном перечне» служит причиной запрета на его интродукцию.

ПЕРСПЕКТИВЫ БУДУЩЕЙ РАБОТЫ И ОБСУЖДЕНИЕ

До сих пор деятельность ЕОКЗР по биологической борьбе концентрировалась на аспектах безопасности интродукции БАББ для окружающей среды и биоразнообразия. В настоящее время в ЕОКЗР рассматривается возможность расширить спектр этой деятельности на разработку стандартов по оценке эффективности БАББ, а также по их применению в подавлении популяций карантинных вредных организмов с целью ограничения их распространения в новые зоны и/или для снижения их вредоносности до приемлемого уровня. Фокусирование на аспектах безопасности наносит вред имиджу биометода в целом, поскольку делает акцент на том, что БАББ могут нанести вред и оставляет в стороне ту пользу, ради которой их интродуцируют. Складывается этакая «презумпция виновности» в отношении БАББ. Если 20-30 лет назад биометод считался наиболее экологичным видом защиты растений, то в последние годы его имидж подвергся серьёзным и, как правило, необоснованным нападкам со стороны средств массовой информации. Появилась даже опасная тенденция полностью запрещать интродукцию неаборигенных БАББ, разрешая использование в биометоде только местных видов. В некоторых странах такой запрет уже принят. Такой подход полностью сводит на нет преимущества классического биометода, направленного на восстановление экологического равновесия, нарушенного в результате проникновения неаборигенного вредного организма в новый регион.

Повышенная вредоносность неаборигенных вредных организмов в новых для них зонах во многом определяется отсутствием их естественных врагов в новом ареале. И если использование БАББ из мест происхождения вредных организмов не может предотвратить их интродукцию в новые ареалы, оно может значительно снизить их вредоносность, в ряде случаев и вовсе делая их не опасными для растений. Тому есть множество примеров в истории применения биометода. «Карантинный вредный организм» определяется в Глоссарии фитосанитарных терминов ФАО (IPPC, 2014) как «вредный организм, имеющий потенциальное экономическое значение для зоны, подверженной опасности, в которой он пока отсутствует или присутствует, но ограниченно распространён и служит объектом официальной борьбы». Организм, который перестаёт иметь экономическое значение в результате интродукции его естественных врагов, перестаёт также отвечать этому определению и, соответственно, не должен больше считаться карантинным. Необходимо признать, что существует риск «отрицательных нецелевых эффектов» применения БАББ. «Нецелевым» можно считать любое воздействие на виды, не являющиеся видами-мишенями конкретной интродукции. Однако трудно определить насколько «отрицательными» такие воздействия могут быть. Так или иначе, ненамеренная интродукция неаборигенных вредных организмов в новые ареалы часто серьёзно нарушает сложившиеся в этих ареалах биоценозы и наносит существенный вред окружающей среде. Завоз естественных врагов этих вредных организмов из зон их происхождения может в значительной степени уменьшить этот вред и восстановить равновесие в экосистемах. При этом новое равновесие во многих случаях отличается от изначального, существовавшего до интродукции вредного организма, но это вряд ли можно считать отрицательным воздействием БАББ.

БАББ также часто используются в рамках интегрированных систем управления вредными организмами. Группа экспертов ЕОКЗР/МОББ могла бы заняться разработками и в этих направлениях. При этом вопросы интегрированного управления вредными организмами попадают под компетенцию Рабочей группы ЕОКЗР по препаратам для защиты растений.

Таким образом, эта группа экспертов может оказаться в ведении обеих рабочих групп ЕОКЗР.

Рассматривается также вопрос о целесообразности развития схемы оценки экологического риска (ОЭР) для БАББ, которых предполагается интродуцировать в новые зоны. В МСФМ 3 (IPPC, 2011) и ряде других документов предлагается использовать схемы АФР для оценки риска, связанного с интродукцией БАББ. Однако это по ряду причин кажется нецелесообразным. Во-первых, схемы АФР разработаны для анализа риска ущерба (в первую очередь экономического), наносимого вредными организмами, и их использование для агентов биометода поддерживает ту «презумпцию виновности», в соответствии с которой они считаются скорее опасными, чем полезными. Во-вторых, ряд разделов схем АФР вовсе не подходит для оценки БАББ, например, оценка вероятности проникновения в зону АФР (поскольку агентов биометода импортируют умышленно), оценка управления фитосанитарным риском, которая заключается в выборе мер борьбы с вредными организмами, и т.д. В-третьих, и это самое главное, оценка риска для БАББ должна быть сравнительной, схема ОЭР должна принимать во внимание возможные риски негативных нецелевых воздействий на окружающую среду в результате интродукции БАББ, но также положительное экологическое воздействие БАББ в результате подавления популяций вредных организмов и сокращения негативного воздействия на окружающую среду альтернативных методов защиты растений, которые должны применяться в отсутствии БАББ (Зайцев, Резник, 2004). Если, например, есть риск, что интродуцированный энтомофаг может помимо основного вида-мишени вредного организма сократить численность нескольких других видов, но принесёт значительно больше пользы окружающей среде (включая восстановление биоразнообразия) за счёт отмены пестицидных обработок и снижения плотности популяций вида-мишени, его интродукция не должна быть запрещена по «экологическим» соображениям. Поэтому разработка специальной схемы ОЭР для агентов биометода со статусом стандарта ЕОКЗР может быть весьма целесообразной. На последнем заседании Совместной группы экспертов ЕОКЗР и МОББ по агентам биологической борьбы в Париже в октябре 2014 года этот вопрос обсуждался вместе с рассмотрением первого проекта такой схемы. В связи с тем, что предлагается проводить сравнительную оценку рисков и положительных эффектов применения БАББ, группа предложила назвать разрабатываемый стандарт не схемой ОЭР, а «Схемой принятия решения об импорте и выпусках в природу беспозвоночных агентов биологической борьбы».

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EU LEGISLATION RELATED TO IPM AND HOW TO AVOID MISTAKES ON OUR WAY TO IMPLEMENT IPM

Sylvia Blümel

Austrian Agency for Health and Food Safety (AGES), Spargelfeldstr. 191,A-1220 Vienna, Austria (http//www.ages.at) & IOBC-WPRS (http//iobc-wprs.org)
e-mail: sbluemel@ages.at, treasurer@iobc-wprs.org

The recently introduced EU-legislation to promote the implementation of more sustainable and environmentally friendly protection of Plant Health poses several challenges to growers in order to meet the legal requirements. Currently existing options and approaches on how to scope with the implementation of the principles of Integrated Pest Management (IPM) are mainly based on the tools and holistic IPM concepts which were originally developed by IOBC-WPRS.

EU-Legislation

Since 2009 four EU-Regulations/Directives[1] concerning the authorization and use of plants protection products (PPPs) have entered into force. Other important regulations (currently under review) in the context of plant health and plant production, especially with regard to the prevention of plant pest introduction, spread and establishment include the EU-Directive 2000/29, the legislation on plant reproductive materials and the Regulation (EC) No 882/2004 on Official Controls including HACCP (Hazard Analysis and Critical Control Points) -principles and QM (Quality Management) requirements. Additionally the Regulation on Maximum Residue Levels of plant protection products (EC 396/2005) or the acts on rural development 2014-2020, the CAP-Farm advisory system (FAS)[2], cross compliance, and European Innovation Partnership (EIP-Agri) will influence the options for growers to apply pesticides in agricultural and horticultural production.

The EU-Directive on the Sustainable Use of Pesticides 2009/128/EC (SUD) has the main objective to further reduce the risks and impacts of pesticide use on human health and the environment with emphasis on the obligatory application of IPM and plant protection approaches or techniques alternative and more innovative compared to those currently in use. The main actions to implement the SUD Directive comprise the development and adoption of the SUD-National Action Plans[3] (NAPs) by the EU Member States (MS) including quantitative objectives, targets, measures and timetables and accompanying measures to achieve the SUD-directive goals. These measures comprise actions such as the training of pesticide users, distributors and advisors, specific information and awareness raising of the general public, the prohibition of aerial spraying, minimizing or banning the use of pesticides, inspection of pesticide application equipment as well as the promotion and obligatory application of the general principles of IPM by growers with the start of 2014.

In Art.14 of the SUD-Directive the major tasks of the MS with regard to the implementation of the Integrated Pest Management principles are stated. The MS shall take all necessary measures and ensure that low pesticide - input pest management (IPM and organic) is promoted with priority to non-chemical methods, wherever possible so that professional users switch to farming practices and products with lowest risk. MS shall establish and support conditions for the implementation of IPM, which include the access of professional users both to information and tools for monitoring and decision

1 Regulation (EC) 1107/2009 concerning the placing of plant protection products on the market, Regulation (EC) No 1185/2009 concerning statistics on pesticides, Directive 2009/127/EC concerning machinery for pesticide application, Directive 2009/128/EC on the Sustainable Use of Pesticides

2 http://ec.europa.eu/agriculture/direct-support/cross-compliance/farm-advisory-system/index_en.htm

3 http://ec.europa.eu/food/plant/pesticides/sustainable_use_pesticides/information_and_awareness_raising_en.htm.

making as well as advisory service on IPM. The MS shall also provide a description on how to implement the General Principles of IPM (Annex III), which are in line with the pertinent IOBC-WPRS publications from the last years. Furthermore MS shall establish appropriate incentives to encourage farmers to implement crop or sector specific IPM guidelines on a voluntary basis (Art. 14, 5).

Various stakeholders have considered the quantitative objectives, targets, measures and timetables to reduce the risks and impacts of pesticides, as well as the indicators to monitor the use of PPPs including timetables and targets for the reduction of pesticide use, as too vague and not clearly formulated, in the way they have been described in several NAPs until the end of 2013.

In order to guarantee the fulfillment of these requirements, comprehensive and regular information exchange is provisioned. The EU-MS have to report about the status of their NAPs implementation to the EC and other MS. Additionally the EC has to report to the European Parliament and the European Council and the Food and Veterinary Office (FVO) will carry out controls and audits. Challenges to meet the SUD requirements for production & processing

Several challenges to meet the legal requirements both on the technical production level as well as on the level of product processing and marketing were identified and should be considered when drawing up future national IPM programs. It should be considered that within a holistic plant production concept integrated pest management represents only one part of the system.

Basic cultivation (plant production) pre-requisites comprise first of all the choice of an appropriate location of the production site and furthermore of the optimum type and quality of the substrate used. Additionally cultivation measures supporting the prevention from or suppression of organisms harmful to plant health include the quality of seeds and planting material and the selection of appropriate varieties, as well as plant nutrition and irrigation. Another major issue is the availability of suitable preventive measures against plant pests, of appropriate forecasting tools and decision-support systems as well as of IPM-suitable PPPs. For greenhouse crops several IPM tools, both preventive and curative are available and can be effectively applied. Important cultivation measures in a broader sense include amongst others the cultivation of tolerant/resistant varieties, greenhouse techniques (construction, material), hygienic measures (removing infested plants/plant material or pest non-crop reservoir plants) and the control of propagation plants for the presence of developmental stages of pests which are not easy to detect at routine visual inspections. As mechanical-physical measures against pest organisms in and on the different plant production matrices on the one hand soil steaming techniques and on the other hand ventilation nets could be applied. Biotechnical tools include coloured sticky traps, pheromone devices, and the direct or indirect use of allelochemicals and of susceptible indicator plants. Biological control comprise the release of BCAs (BioControl Agents) partly also in combination with induced resistance for plant pathogens. Additionally chemical measures can be applied, if selective and BCA compatible PPPs are available and suitable application technique are used.

In field crops, especially in arable crops, one of the most important preventive measures to implement IPM and SUD is the crop rotation, which poses a challenge especially for small and medium size farms. This is due to the economical market pressure which derives from the required crop specialization and partly needed expensive high tech equipment, but also from resulting in higher training efforts as well as from the dependence of the growers from few transnational retailers. To scope with this situation as options (more) co-operation, the production of "new" crops and direct marketing have been proposed.

Other important preventive measures are forecasting tools and decision support systems, for which as most urgent needs within the EU the development, improvement, harmonization, standardization and validation of forecasting models and tools were identified together with a focus on regional and cross border co-operation as a priority.

The reduced availability of IPM-suitable PPPs pose an additional challenge for growers as on the one hand the pesticide portfolio variability is decreasing (due to the re-evaluation process of PPP's in the EU), which might lead to an increasing occurrence of pest resistance to pesticides. On the other hand also the availability of alternative PPPs such as Low Risk (LR) substances and Bio Control Agents (BCAs) is limited. For (LR) substances currently no specific criteria for their identification or a specific modus for their approval will be applied, although a fast track, tailor made process is considered as desirable by different stakeholders. Besides the availability of e.g. BCAs is dependent on various "regulatory" factors for access, import and release which are stated mainly in the Nagoya protocol on Access & Benefit Sharing (ABS), as a result of the Convention on Biological Diversity (CBD)1992. In the ABS as principle "the fair and equitable sharing of the benefits arising out of the utilization of genetic resources" is laid down, as the genetic resources are owned by the source countries. Other regulations applied are the ISPM No. 3

"Guidelines for the export, shipment, import and release of Biocontrol Agents and other beneficial organisms (2005)" and the EPPO guideline 6/2 "Guidelines for Import and release of non-indigenous biocontrol agents" (2010).

Challenges to scope with phytosanitary pests in context with SUD

With regard to phytosanitary pests, the application of IPM could present an important supplementary or pesticide replacing option for the containment and control of the increasing number of new invasive or (re) emerging pest species, due to globalization and global change, as the spread and establishment of those pests could be delayed or even inhibited. For several pests of phytosanitary importance IPM tools such as prevention and cultural methods or the use and promotion of plant pest antagonists or induced resistance represent the major if not the only measures to scope with the pests in a feasible way. Crop rotation is the only feasible methods to suppress persistent developmental stages of phytopathogenic fungi and nematodes in the soil as well as cultural methods or BCAs for the suppression of several arthropods pests or invasive plants.

The four major steps of protective measures against invasive pest species, which are prevention, eradication, containment and control are assisted by several measures required for the implementation of the SUD directive such as, the application of IPM as part of the basic production principle, the increased capacity building, the obligatory monitoring in production with low pesticide input and research for alternatives to„ conventional" plant protection tools. On the other hand the prohibition and/or /restriction of the use of PPPs in sensible areas and the prohibition of specific application techniques might be detrimental to the required control effect.

Socio-economic challenges to meet the SUD requirements

The socio-economic challenges derive from the contradiction between the consumer expectations and demands and the current situation and the options for the producers. Consumers expect no or at least low pesticide residues on food and require a low risk of food, water and environmental pollution by plant protection. At the same time a contribution of farmers to sustainable food production (food security) and to the protection of biodiversity is expected. However plant products produced according to IPM standards mostly cannot be marketed differently from "conventionally" produced products up to now. Still no harmonised IP lables or certification schemes exist and different "no residue" programs of retailers are applied, partly within "Global gap" requirements, which pose considerable challenges both to growers and consumers. Benefits of IPM such as high quality (certified) products should be made visible for consumers as well as the IPM-products themselves, which should be labelled specifically for better recognition. IPM production guidelines should allow for economic feasibility and appropriate income for the producers. Thus, coherence of IPM production requirements with the demands of retailer organisations should be sought and start incentives for producers from public programmes considered.

Challenges for Stakeholder Involvement & awareness raising

All stakeholders, the farmers, farmers associations, the NPPOs including the NRL's (national Reference Laboratories), extension & advisory services as well as research funders and research providers and also NGOs and consumer organisations should be included in a public discussion and awareness raising process. This process could use different means of communication, such as round table meetings, workshops, demonstration farms and different media at an early stage during the development and the implementation process of IPM.

Challenges for Capacity Building, Training and Transfer of Know-how

Quality assured capacity building and transfer of know-how are outlined as indispensable requirements in Art. 5 of the SUD-directive. The MS shall establish and nominate official training bodies, which are authorized to carry out the obligatory, certified training for distributors, advisors and professional users (farmers) of pesticides. In this context requirements and procedures for granting, renewal and withdrawal of certificates (Art. 5, 3) are specified. The training shall be regularly updated with scientific and technical progress knowledge (Annex I, 4). National IPM R&D (research and development) programmes should be installed to create knowledge taking into consideration the national or local peculiarities as input for integrated production guidelines and for training measures. The MS shall develop crop specific holistic integrated production guidelines and training.

Available information and tools to scope with the implementation of the principles of Integrated Pest Management

Substantial information is already available through the IPM concepts of IOBC-WPRS providing information both on the principles and the implementation on farm-level and through the experience since 20 years with IPM and Agri-Environmental Programs which were developed and adapted over time in several MS.

A modern holistic concept of and various tools for IPM as part of a sustainable systems approach of Integrated Production (IP) were first developed in the late 1980s by the International Organization for Biological and Integrated Control (IOBC). Regularly updated information for advisors, producers and other interested stakeholders, such as the Integrated Production Principles of IOBC, Crop specific Integrated Production Guidelines, the IOBC IP Tool Box and the IOBC Pesticide Side Effect Database can be found on the IOBC-WPRS homepage:

http://www.iobc-wprs.org/ip_ipm/index.html

http://www.iobc-wprs.org/ip_ipm/IOBC_IP_principles.html

http://www.iobc-wprs.org/ip_ipm/IP_guidelines_crop_sprecific.html

http://www.iobc-wprs.org/ip_ipm/IOBC_IP_Tool_Box.html

http://www.iobc-wprs.org/ip_ipm/IOBC_endorsement_procedure.html

http://www.iobc-wprs.org/ip_ipm/download_documents.html

The booklet Integrated Pest Management - Design and application of feasible and effective strategies (Wijnands et al. 2012), compiles in a condensed form the IOBC expertise on IPM.

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ADDITIONAL GLOSSARY

EC (2009): COUNCIL DIRECTIVE 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community

ISPM No.3 Guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms. International Standard on Phytosanitary Measures established under the IPPC : https://www.ippc.int/en/publications/600/ https://www.ippc.int/en/core-activities/standards-setting/ispms/#publications

CBD: Convention on Biological Diversity http://www.cbd.int/

ABS: Access and benefit sharing http://www.cbd.int/abs/

CP: The Cartagena Protocol on Biosafety to the Convention on Biological Diversity http://bch.cbd.int/protocol

EPPO: http://www.eppo.int/

http://www.eppo.int/PPPRODUCTS/information/new_eu_regulations.htm

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RATIONAL USE OF ENTOMOPHAGOUS IN COMPLIANCE WITH THE REQUIREMENTS OF MODERN GREENHOUSE CROP PRODUCTION AND ENVIRONMENTAL LEGISLATION IN RUSSIA

Natalia Beliakova

All-Russia Institute of Plant Protection, St.-Petersburg, biocontrol@vizr.spb.ru

ABSTRACT

The last decades were characterized by significant changes in the species diversity of insects, and in natural habitats and agrocenoses a change of the dominant species and the increased frequency of biological invasions are noted. Against new invasive pests should be used polyphagous predators, as there are no specialized predators in the native fauna. Signs, determining effectiveness of polyphagous predators in pest control, are often related with its high invasion potential. Because of it, as promising biocontrol agents we select from the natural environment species that are potential inviders. In recent decades, frequency of cases of entomophage invasion, introduced earlier for biological plant protection, has increased. In some cases invasion activity of former agents of biocontrol caused negative consequences, including replacement or disappearance of several indigenous species, which previously occupied the dominant or subdominant position in the species communities. Among polyphagous predators the Multicolored Asian lady beetle *Harmonia axyridis* (Coccinellidae) is a demonstrative example of introduced entomophagous, which became invider. In this regard, one of the important aspects of the work on development of entomophage bioresources is their invasive potential evaluation. In the present work attempted to define features that distinguish potential inviders of promising biological control agents. Screening was based on the collection VIZR`s entomophages. As a result, it is shown that a critical mass of pupation; size sexual dimorphism and intraspecific variation in the weight can be used to estimate of invasive potential.

Key words: introduction, acclimatization of entomophagous, biological control, invasion potential, Coccinellidae, *Harmonia axyridis*, ***Cheilomenes sexmaculata, Propylea dissecta.***

INTRODUCTION

The size and isolation degree of the modern industrial hothouses make them similar to the island ecosystems. The plants are cultivated on the area of 5-10 ha, in isolation from the external environment (ventilation system and the entrance are screened). However, the hothouse as an «island» biotope, human-created and maintained, has significant difference from the real island ecosystems. Hothouse communities are unstable. These anthropogenic «islands» are in state of continuous dynamic changes resulting from lasting activity of man who has created them. In particular, hothouses are regularly flooded by entomophages without reference to presence or absence of their preys-phytophages. Sustainable development of the hothouse agro-ecosystems requires maintenance of the biocenotic balance, preservations and activation of the useful biota, the most important component of which are predatory and parasitic insects – entomophages.

The model of island biogeography (MacArthur, Wilson, 1967) is hardly applicable to the hothouse. Species complexes that are formed in the hothouse will always differ from the natural communities. Hothouse

«island» is originally constrained. From the moment of planting man purposefully fills hothouse with predators and parasites. Therefore, any entomophage-invader faces pressure of competitor and food stress from the first day. So, we suppose that in the modern industrial hothouses species of entomophages, resistant to food stress and competitive replacement, will primarily survive.

Apart from large-scale implementation of the industrial technologies of glasshouse cropping there is another factor that can cardinally change the further of biological control in the field of entomophage bioresources development. These are increasing requirements of the environmental legislation, which restricts introduction of entomophages outwards their native habitat (Anonymous, 2011).

Nagoya protocol and Convention on conservation of biodiversity order to evaluate and assume proper measures (for example, to develop guidelines or codes of practice, pertinent to biological control agents marketing and utilization) on national, regional and global levels aiming to eliminate potential risk of transformation of the biological control agents to the invasive foreign species. These restrictions were imposed mainly due to the fact that the last decades were characterized by significant changes in species diversity of insects; in natural biotopes and agroecosystems the change of the dominant species takes place and the frequency of biological invasions steadily increases. The main causes of these processes are globalization, anthropogenic transformation of natural landscapes and agricultural lands, the introduction of intensive crop production, and the creation of new habitats (including industrial hothouses), intentional introduction and accidental importation of insects.

Increase in volumes of crop production import, as well as seed and planting material, promotes accidental importation of insects, including dangerous pests.

Invaders destroy system of biological protection. Fighting with western blossom thrips, cotton whitefly *Bemisia tabaci* Genn., South American leaf miner *Liriomyza huidobrensis* Blanch. and tomato leaf miner *Tuta absoluta* Povolny (Meyrick), early in the invasion cycle the agricultural producer had to conduct chemical treatments. And only some yeas later success was achieved in selection of entomophages that partly «filled the hole», made by the given invasive pests in the system of biological control.

For fast response to the increase of frequency of dangerous pest invasion it is appropriate to form pool of entomophages with broad food specialization – «universal soldiers» of biological control. Against new pests, the application of polyphagous predators is promising at the first stage of invasion, unless specialized entomophages, which are usually absent in the domestic entomofauna, are introduced.

In addition to invasion, there is another factor, overcomplicating phytosanitary situation in the modern hothouses. This is widespread adoption of the intensive agrotechnologies crop growing (photoculture, extended cycle, inplanting – piecemeal replacement of plant in the course of culturing). All this provides high crop yield, but at the same time requirements to entomophages, for the most part to their search activity and plasticity, are substantially increased.

With high crop yield, characteristic for the intensive crop production, the economic harmfulness threshold tends to zero. For example, in the extended crop cycle using inplanting technology, the average crop yield of cucumber tends to 70 kg/m^2. The margin for error during implementation of protective measures grows in proportion to the crop yield. Under such conditions preventive entomophages colonization becomes basic cost-effective protection method. It allows controlling pests at the starting low population level, when biological protection is maximally effective.

The objective of this study is to mark out main qualities of entomophages, defining their effectiveness in case of preventive colonization in conditions of the glasshouse cropping.

MATERIALS AND METHODS

Screening of potentially suitable predatory species with broad food specialization and optimization of the entomophages complex were carried out on the basis of the collection of Coccinellidae, formed in VIZR. As a model group, the lady beetles were chosen, because they show significant diversity of morpho-ecological adaptations to insect feeding. Good deal of bioresources of Coccinellidae-polyphages remains undeveloped. In the world practice Coccinellidae-oligophages, specialized in one particular group of pests, are primarily used. So, it is likely to find universal species that can be used for broad-spectrum pest control among lady beetles.

In our work laboratory populations of entomophages from the collection of VIZR, including 8 species of the lady beetles from three dimensional classes, were used:

1) small (average weight of imago is up to 15 mg): *Propylea japonica* (population origin is Ussuriysk, 2012), *Propylea 14-punctata* (Saint-Petersburg, 2014), *Propylea dissecta (*Nepal, Chitwan, 2013), *Cheilomenes sexmaculata (*Nepal, Chitwan, 2013), *Cycloneda sanguinea limbifer* (Cuba, 1972),

2) middle (15-30 mg): *Harmonia axyridis (Serbia, 2013; Sochi, 2012; Alma-Ata, 2014; Irkutsk, 2012), Harmonia 4-punctata (Serbia, 2013).*

3) large (more then 30 mg) *Harmonia dimidiata (*China, Guangzhou, 1990; Nepal, Pokhara, 2013).

RESULTS AND DISCUSSION

Analysis of the characteristics of vegetable and flower crops cultivation using low-capacity technology allows us to identify key qualities of entomophages that determine their efficiency in the modern hothouses.

1. The entomophage must have high dispersal ability. This requirement is conditioned by the necessity to reduce work effort for entomophages introduction, reaching 20 man-hours per hectare in case of manual dispersal. For separate species use of mechanical appliances (sprayers or devices for scattering of entomophages) is suitable. In most of cases mechanized dispersal leads to injuries of biomaterial and loss of its efficiency. Therefore, the optimal way is liberation of individuals, able to fly; it reduces work effort, does not injure insects and allows using their search activity for opportune suppression of the primary foci of pests.

Adult predators are characterized by their high mobility and search activity caused by hunger (as larvae), as well as reproductive instinct. In modern hothouses entomophages on imaginal stage stand a better chance to reveal the focus of pest, than person. The reason is substantial density of planting (for tomato and cucumber it is 3-4 stems/m^2) and height of protected plants up to 3 m (fig. 1). Yellow and blue glue traps give warning of pest appearance, but they do not give precise information about its localization in the hothouse. As a result the person generally reveals foci by the injuries of plants, in other words with delay, when the pest is already accumulated. It means that winged entomophages, able to find single pests, are necessary, especially in the hard-to-reach top level (above 2.5 m) that is actively populated by sucking pests.

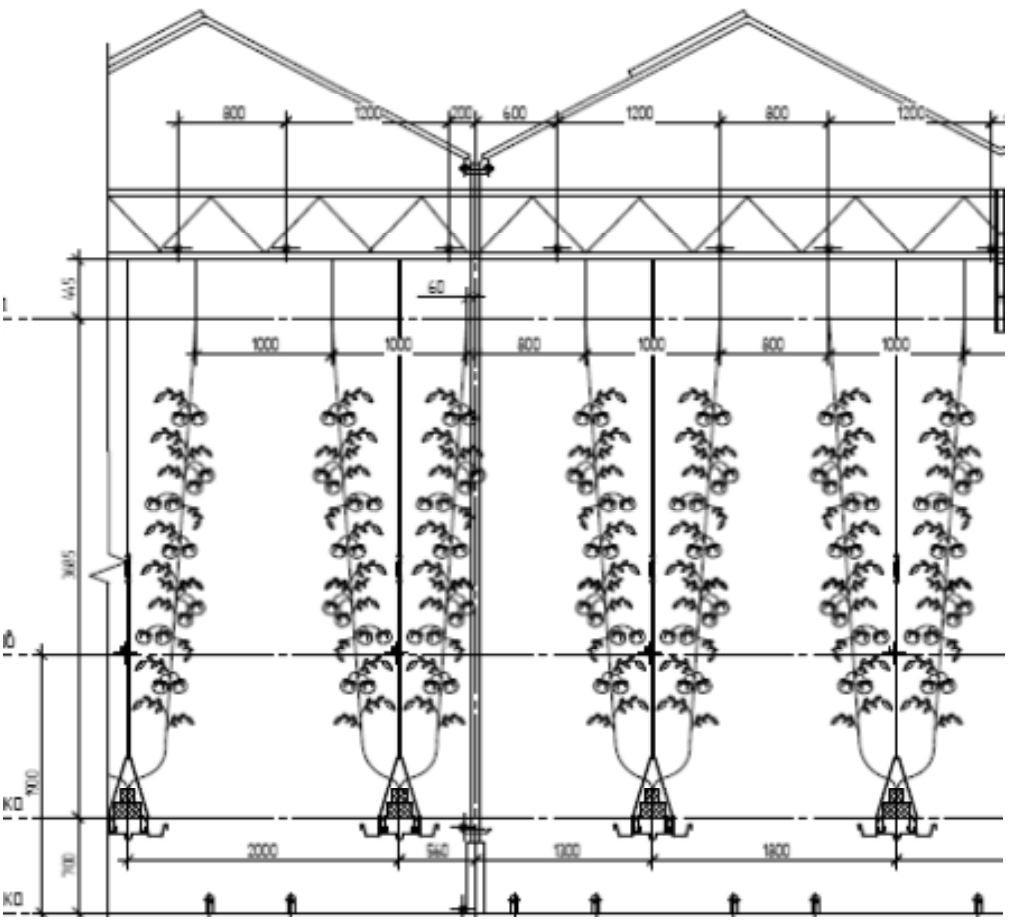

Figure 1. Scheme of planting of tomatoes in case of use of low-capacity technology in the hanging gutters (trays) (CJSC «Oldeyevskaya», 2013)

When cultivating leaf vegetables and flower crops on the hydroponic automated lines, access to plants is only possible on the perimeter of the shelving units. For example, in case of assembly line and cassette production of leaf vegetables and radish the hydroponic shelving units with operating area more then 300 m^2 that is not divided by passages, are used. Because of it, monitoring with the use of traditional methods (visual examination of plants) is complicated, and local introduction of the entomophages to the foci of pests is impossible.

Tomato, cucumber and pepper are grown on mineral growing medium in trays, hanged at a height of about 1 m. Entomophages on imaginal stages, fallen from the plant, can come back, while wingless insects (larvae of bugs, beetles of midges, Phytoseiidae ticks) remain on the floor of the hothouse and, as a rule, perish in the absence of food. Therefore, one of the important qualities of entomophages, determining its effectiveness, is high mobility, including the ability to fly.

2. The entomophage has to survive in hothouse a long time (1-2 months) in the absence of target pest, feeding on substitute feedstuff (eggs of lepidopterans, copepods, carbohydrate feeding, and artificial growth media). This requirement was stated on the principle that preventive introduction is the main method of entomophages application in the modern phytosanitary technologies.

Specialized entomophages (olygo- and monophages) perish in the absence of the host or prey during 7-10 days. Regular introduction with work effort up to 20 man-hours per hectare is necessary. Polyphagous predators can survive in agrocoenosis a long time (up to 2 months) when feeding. It is preferable to use those species, in which natural feed substitute induces food diapause, so that the predator does not spend the reproductive potential in the absence of the target prey-pest.

Substitute feedstuff allows accumulating predators in areas, where appearance of pest is the most likely. For example, in case of interplanting technology use, young plants are regularly integrated to the hothouse during 12-18 months. Piecemeal replacement of plants that complete period of bearing, takes place. Old plants are replaced by younger ones, attracting pests, accumulated in the hothouse earlier. This is one of the methods of intensification of hothouse vegetable growing. On the one hand it increases the risk of mass reproduction of phytophages, from the other hand it extends possibilities of seasonal colonization of entomophages, for which young plants are reservation for accumulation and preservation in the hothouse.

High risk areas in the hothouse are not only young plants, but unstable varieties plantations that are grown together with stable ones. Cultivation of several varieties with different stability level in one hothouse is widely used when growing flower crops (for example, roses for cutting). Local accumulation of phytophages on the areas with varieties that are susceptible to injuries, takes place. Additional nutrition of entomophages on unstable varieties plantations allows attracting and keeping predators on such problem areas of the hothouse that stabilizes phytosanitary situation.

Biological protection stability is especially actual under the conditions of the extended crop cycle, as well as for perennial crops (flower, small-fruit crops), when the crop vegetation terms range from 10 months to 5 years, and regular application of chemical protective agents can cause resistance to emerge.

Guided by the above-described requirements that intensive crop production impose to the entomophages, we have defined screening criteria. It is advisable to select those species that (1) have broad spectrum of preys from different systematic groups, and that (2) are suitable for long-term colonization of the imaginal stage when feeding substitute feedstuff (eggs of grain moth) in the absence of the target species of pest.

Bioresources of polyphagous predators are rather extensive. In the domestic fauna there are dozens of thousands of species within the limits of order Coleoptera, Hemiptera and Neuroptera, and in the world fauna this number is 5-6 times more. By now more than 100 species of polyphagous predators were tested for the biological control, and about 20 of them are actively used by method of seasonal colonization for protection of vegetable, fruit, small-fruit and flower crops.

Russians agricultural producer uses primarily specialized predators – midges *Aphidoletes aphidimyza* Rond. and ticks *Phytoseiulus persimilis* Athias-Henriot, more rarely bugs-polyphages – plant bug *Macrolophus nubilus* Herrich-Schaeffer and Anthocoridae of genus *Orius*. Predators from the order Coleoptera are used in very rare cases, generally Coccinellidae on salad lines.

Good deal of bioresources of Coccinellidae-polyphages remains undeveloped. In the world practice Coccinellidae-oligophages, specialized in one particular group of pests, are primarily used. *Cycloneda sanguinea limbifer* Casey, *Harmonia dimidiata* Fabr. are used for plant lice control, *Delphastus catalinae* Horn – for white flies control; *Cryptolaemus montrouzieri* Muls. – for management of scale insects and pulvinaria, *Chilocorus renipustulatus* Scr. – for parlatoria control, *Stethorus punctillum* Weise - for red spider control (van Lenteren, 2012). Taking into account significant diversity of morpho-ecological adaptations of lady beetles to insect feeding, it is evident that development of natural resources of entomophages of this systematic group

should be continued. It is highly probable to find among the lady bugs suitable universal species that can be used for control of broad spectrum of yet existing pests, as well as potential invaders, whose appearance we can predict on the territory of the Russian Federation in the coming years.

In our work laboratory populations of entomophages from the collection of VIZR, including the species of lady beetles from genera *Propylea* and *Harmonia*, were used. The morpho-ecological criteria for the evaluation of biotechnological potential of the lady beetles were revealed. It was shown that the manifestation characteristics of dimensional sexual dimorphism and the extent of intraspecific weight variability under the conditions of food stress can be used to screen promising species-producers among the representatives of the family Coccinellidae.

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INTEGRATED WEED MANAGEMENT IN FIELD CROPS: SUSTAINABILITY AND PRACTICAL IMPLEMENTATION

Goran Malidža[1] and Sava Vrbničanin[2]

[1]Institute of Field and Vegetable Crops, Novi Sad, Serbia
[2]University of Belgrade, Faculty of Agriculture, Belgrade-Zemun, Serbia
sava@agrif.bg.ac.rs

ABSTRACT

Integrated Weed Management (IWM) is a sustainable approach to the management of weeds by combining all available weed control techniques, including preventative measures, monitoring, crop rotations, tillage, crop competition, mechanical and physical control, herbicide rotation, herbicide mixtures, biological control, nutrition, irrigation, burning, etc. in a way that minimizes economic, health, and environmental risks. The first step in IWM program is to monitor the fields for signs of weed infestation or potential weed problems. Proper weed management involves a thorough survey of each field after crop harvest to identify major weed species in the field. When an annual crop (maize, sugar beet, soybean, wheat, etc.) is to be grown in the field in the following year, this information is used to assess the importance of each weed species and to select the appropriate management strategy to be used for the coming crop. In annual crops, fields are also monitored after the crop has emerged, to assess the effectiveness of the selected management alternative and whether additional management measures are needed. For planting perennial field crops, such as alfalfa, an assessment of weed species composition is conducted after harvest of the previous crop, to determine the appropriate management alternative to be used during the establishment. In an established crop, fields are monitored to determine the need for additional measures to manage annual, biennial and perennial weed species.

Cultural practices in the control of weeds include anything which makes the crops more competitive against them: proper seedbed preparation, planting time, fertilization, crop rotation, row spacing, seeding rate, and variety selection. Mechanical weed control includes the use of pre-plant tillage such as ploughing, disking, and field cultivating. These primary and secondary tillage systems can help reduce the rate and spread of certain perennial weeds such as *Agropyrum repens, Sorghum halepense, Taraxacum officinale*, etc. After planting operations such as rotary hoeing, row cultivating, flaming and hand hoeing can help reduce the dependence on herbicides. Finally, herbicides should provide a convenient, economical and effective way for the management of weeds. They allow the fields to be planted with less tillage, allow earlier planting dates, etc. Herbicides may not be a necessity on some farms (organic agriculture), but without the use of chemical weed control, preventive, mechanical, physical and cultural control measures become that much more important. When choosing a herbicide program, the decision should be based on potential weed problems, crop and herbicide rotation, injury potential, tillage system and available application equipment, soil texture and organic matter, potential environmental hazards, and cost. Herbicide rotation is an important management consideration. Rotating herbicides reduces the risk of developing herbicide-resistant weeds. Other tactics that help prevent the development of resistant weeds include: using herbicide mixtures that contain more than one herbicide class;

using shorter soil residual materials, including non-chemical control measures; avoiding spreading resistant weed seed with machinery or in manure; and helping destroy weed-seed-infested forage by ensiling.

This higher level of complexity partly explains why IWM has not received the same attention as integrated management of other pests. Adding to the complexity is that most non-chemical tools are not as effective as herbicides, i.e. they cannot be considered as stand-alone methods but have to be combined with other methods in a systematic way to provide sustainable and reliable weed control. Finally, some non-chemical weed management options incur an additional cost that needs to be balanced against the potential long term benefits of more sustainable IWM strategies.

Key words: integrated weed management, sustainability, implementation

INTRODUCTION

Weeds are troublesome in many ways, because they reduce crop yield by robbing them of light, water, soil nutrients and space (Ghersa et al., 2000). Also, weeds can produce allelopathic substances that are toxic to crop plants (Jabran et al., 2015). Weeds often serve as hosts for crop diseases and optimal places for diseases to overwinter. Some weeds, such as *Agrostemma githago*, *Avena fatua, Cuscuta campestris* and many others also reduce the crop quality. Because of this and the current practice, the future of sustainable weed control must be based on the implementation of the principles of Integrated Weed Management (IWM). Consequently, IWM strategies are focused on:

- Limiting weed establishment in the crop from the soil seed bank or subterranean vegetative organs such as roots, rhizomes, bulbs, tuber-bulbs, etc. (Clements et al., 1996);
- Limiting competition for resources such as light, nutrients and water by removing weeds or manipulating the weed flora to reduce their competitive impact (Röhrig & Stützel, 2001; Chauhan & Abugho, 2013);
- Limiting the return of seeds or their vegetative organs to the soil seed/vegetative organ bank (Benech-Arnold et al., 2000).

An IWM strategy attempts to achieve one or more of these goals and this framework shoud assess the sustainability and resilience of IWM strategies. Therefore, IWM is a sustainable approach to managing weeds by combining all available weed control techniques, including preventative measures, monitoring, crop rotations, tillage, crop competition, mechanical and physical control, herbicide rotation, herbicide mixtures, biological control, nutrition, irrigation, burning, etc. in a way that minimizes economic, health, and environmental risks (Swanton & Murphy, 1996; Vrbničanin et al., 2006; Wilson et al., 2009; Peshin & Pimentel, 2014). Because the available techniques typically have lower individual efficacy than herbicides, IWM requires the combining of different measures. It is unlikely that a single control measure on its own will be effective in the long run. The concept of IWM is to maintain balanced weed flora and to reduce the reliance of cropping systems on herbicides, by adopting all available tools for the decrease of weed pressure and competition. Consequently, IWM has been referred to as "many little hammers" in the modern cropping practices.

Basic Principles and Reasons for the Implementation of IWM

The concept of IWM has been proposed as a component of Integrated Pest Management (IPM), a crop production paradigm in-between conventional agriculture and organic farming (El Titi, 1992). The objectives of IWM-based systems are to reduce the reliance on herbicides by adopting agronomic measures: (1) reduction of weed seed banks in the soil (2) decrease of the density of weeds emerging in crops, (3) reduction of their relative competitive ability, and (4) control of emerged weeds using non-chemical techniques (Pardo et al., 2010). Furthermore, in the modern agricultural practices there are more reasons why the IWM system is the most appropriate long-term strategy for weed control, such as: (1) the increasing concern for the effects of herbicides on human health and environment, (2) the development of herbicide resistant weeds, (3) weed shifts, (4) invasive weeds and climate change, (5) the slow development of new herbicides, etc.

Finally, in the past two decades weed management has become a key issue for European agricultural practices due to following reasons: (1) frequent herbicide treatments in most crops throughout Europe, except, of course, in organic farming, (2) herbicides are

the pesticide residues most frequently found when analysing the quality of surface and ground-waters, (3) the development of weed populations resistant to the most frequently used herbicides has become a real threat to the sustainability of current chemical weed control strategies, (4) the increase in cost of chemical crop protection, due to the withdrawal of several old and cheaper herbicides (Ramesh, 2015). Therefore, these are key points for implementing innovative strategies which focus on lower pesticide inputs and combine all available weed control techniques within the IWM concept.

Networking Research and the Main Factors for Successful IWM

The development of an IWM system must take all aspects of the cropping system into consideration. Generally, each cultural practice influences the competitive ability of both the crop and the weed community, leading to a multitude of complex interactions. However, efforts must be made to work within the existing production practice to ensure a greater likelihood of acceptance by the cropping community. Thus, it is important to change the existing system in a progressive manner. This progression must be reflected in the research strategy. According to Swanton & Weise (1991) this would allow for the transfer of specific components through education and extension, while research continues to refine and further develop the system (Figure 1).

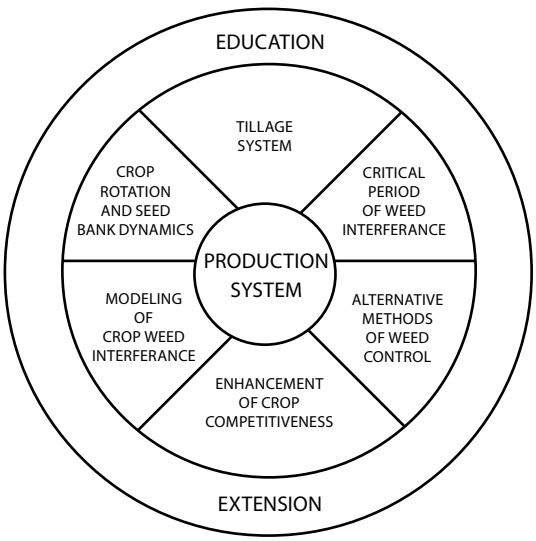

Figure 1. Research strategy for the development of an integrated weed management system (Swanton & Weise, 1991)

The different components of IWM, such as crop selection, crop husbandry, plant nutrition, crop protection, farm hygiene, and the site-specific conditions, all are factors which influence the successful adoption of the basic IWM concept. Farmers' field activities, directly or indirectly, influence weed growth in almost every phase during the vegetation period. According to Zoschke & Quadranti (2002) major factors affecting weeds and consequently weed management efficiency are summarized in Figure 2. Crop selection, crop husbandry, plant nutrition, crop protection, and farm hygiene are all factors which, in one way or another, have been demonstrated to affect the germination and development of weeds, as well as weed population dynamics. Additionally, the site specific conditions ('location') are of major importance (Zoschke & Quadranti, 2002).

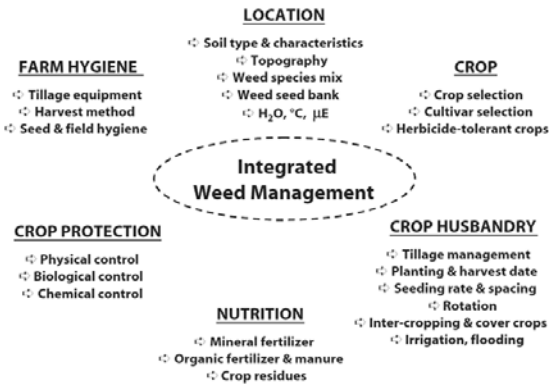

Figure 2. The main factors affecting weed management efficiency (Zoschke & Quadranti, 2002)

Preventive Practices

Generally, the best start of any weed management program is to reduce the potential for weed seeds introduction into the field. Preventive practices may include many activities such as: (1) avoiding introduction of new weed species and where possible preventing the introduction of endemic weed seeds in inputs such as manure or compost, (2) control of weeds in the field, before they have the chance to set seeds, (3) control of weeds in the field margins to prevent the entry of weed seeds into the field, (4) planting of certified crop seeds, (5) controlling volunteer weeds and patches of new species or herbicide-resistant weeds, (6) cleaning equipment (especially tarping grain trucks), (7) using well-composted manure, and etc. (Knezevic, 2014).

Weed Monitoring

The first step in an IWM program is to monitor fields for signs of weed infestation or potential weed problems. Proper weed management involves a thorough survey of each field after crop harvest to identify major weed species in the field. When an annual crop (maize, sugar beet, soybean, sunflower, wheat, barley, etc.) is to be grown in the field the following year, this information is used to assess the importance of each weed species and to select the appropriate management strategy to be used for the coming crop. In annual crops, fields are also monitored after the crop has emerged to assess the effectiveness of the selected management alternative and whether additional management measures are needed. For planting perennial field crops, such as alfalfa, an assessment of weed species composition is conducted after harvest of the previous crop, to determine the appropriate management alternative to be used during the establishment. In an established crop, fields are monitored to determine the need for additional measures to manage annual, biennial and perennial weed species.

Weed Seed Bank Management

A soil seed bank includes all viable seeds and vegetative propagules present on and in the soil which might have originated from the recent seed rain of previous years (Shrestha et al., 2002). Therefore, in principle, weed seed bank management can be integrated into a strategy for the control of weed aboveground infestations. Weed species abundance and diversity determine the structure of the weed seed bank in arable lands (Bellinder et al., 2004). Soil seed bank populations are significantly influenced by both crop rotation and tillage type (Ball, 1992; Blackshaw et al., 2001). However, crop rotation is more influential than any other practice (Cardina et al., 2002). Crop rotation creates a higher possibility for weed mortality, when compared to monoculture (Martin & Felton, 1993). Also, variation in crop sequences can increase weed emergence, establishment and seed production (Dorado et al., 1999). Understanding the influence of crop rotations and their companion impacts on weed seed bank provides helpful information to improve decision making systems (Hosseini et al., 2014). Additionally, for weed seed bank management, agricultural engineers from the University of South Australia in collaboration with AHRI (Australian Herbicide Resistance Initiative) are applying the "Harrington Seed Destructor" known as the Integrated Weed Destructor (IWD). It has been widely acknowledged by many in the agricultural industry that weed seed destruction at harvest is necessary as a key non-herbicide weed control tool to manage herbicide resistant weeds (http://ahri.uwa.edu.au).

Innovation in Mechanical and Physical Weed Management

As a consequence of the EU pesticide policy, in addition to national pesticide action plans, many herbicides have been withdrawn from the EU market (Jensen et al., 2014). Non-chemical methods will be necessary to fill the gaps where herbicides are no longer available or where those approved do not cover the spectrum of weed species causing problems. When compared with herbicides, mechanical and physical weeding practices such as weed harrowing, hoeing, disking, brush weeding, torsion and finger weeding or flaming are usually less effective, both in the short and long term (Melander et al., 2015). But, inter-row cultivation is commonly employed in row crops, in both conventional and organic farming (Malidza et al., 2009). Also, primary and secondary tillage can help reduce the rate and spread of certain perennial weed species such as *Agropyrum repens, Sorghum halepense, Taraxacum officinale*, etc. (Conn, 1987; Carter et al., 2002).

In the past decade, especially in organic farming, flame weeding has shown to be particularly promising. The advantages of flame weeding are that it leaves no chemical residue in the soil and water and does not disturb the soil, however, its disadvantage is its high consumption of costly fossil fuels (Ascard, 1998; Datta & Knezevic, 2013). Flame weeding is an acceptable weed control option in both organic and conventional production systems. Flaming is used mostly as one part in a weed control process that involves other methods that are usually applied later (Knezevic et al., 2013). Pre-emergence flaming, followed by post-emergence brush weeding have been found to be particularly promising. Also, hoeing close to the row may be as good as brush weeding in some situations (Melander & Harvig, 1997).

Crop competitiveness

Field studies showed that enhancing crop competitiveness by planting competitive varieties at relatively high seeding rates and through strategic fertilizer placement including sub-surface banded or point-injected nitrogen can reduce the impact of weeds on the crop yield and the amount of weed seed entering the soil seed bank (O'Donovan et al., 2007; Vrbničanin et al., 2012). Enhancing crop competitiveness also improves herbicide performance, especially when

herbicides are applied at reduced doses. Crops differ in their competitiveness with weeds, based on their emergence, leaf-area expansion, light interception, canopy architecture, leaf-angle, shape and competitiveness (Isaac et al., 2013). Within a crop species, cultivars may vary in their competitiveness. While the improved varieties may be high yielding, the traditional varieties exhibit multiple adaptations, competitive ability against weeds and require less agricultural input. The use of competitive crops to discourage weeds is an important IWM strategy. To maximise the crop production, by minimising the impact of weeds, replacement series and additional series designs have been recommended for intercrop, cover crop and green manure selection (Maxwell & Donovan, 2007).

Cover crops

Cover crops can be very effective in suppressing weeds. Cover crops may be sown into extant crops, or the crop residue left after harvest, to reduce the time when weeds grow without competition from the crops (Swanton & Murphy, 1996). A cover crops' biomass and canopy helps it compete with weeds (Liebman & Davis, 2000). There are at least two major types of cover crops that can be used for weed control: (1) off-season cover crops and (2) smother crops (a cover crop grown during parts or all of the cropping season) (Buhler, 20002). When using off-season cover crops, the goal is to produce sufficient plant residue to create an unfavorable environment for weed seed germination and establishment. When using a smother crop, the goal is to displace weeds from the harvested crop through resource competition. Furthermore, cover crops may reduce soil erosion and improve soil structure and nutrient cycling (Wagner-Riddle et al., 1994).

Site-Specific Weed Management

Information and technology based agricultural management system are used to identify, analyse, and manage spatial and temporal variability within the fields, for optimum profitability, sustainability, and environmental protection (Robert et al., 1994). Although weeds are not uniformly distributed across the fields, most weed control practices are applied uniformly. The uniform application of herbicides over non-uniform weed populations was identified as an important source of inefficiency in weed management (Cardina et al., 1997). Site-specific weed management may result in reductions of herbicide quantities used and ecological and economic benefits.

Major limitations with mechanical weeding include limited weed control in crop rows at early, vulnerable crop stages, weather-dependent effectiveness, and difficulties in handling crop residues. Precise steering and depth control, improved seedbed friability and lighter tractors or controlled traffic could bring considerable improvements. To expose weed seeds to predators, position them for fatal germination, viability loss or low emergence may require completely different soil displacement patterns than those of current implements and systems. Controlled traffic and precise strip tillage offer good opportunities for implementing these weed management strategies in minimum-tillage systems (Kurstjens, 2007).

GPS technology and GIS software methods are widely available commercially and have been used by weed scientists in the manual development of geo-referenced maps of weed distributions in agricultural fields. When integrated with machine vision, the weed sensing technology allows for the automatisation of this valuable management tool. Despite these challenges, there have been few completely robotic weed control systems demonstrated in the agricultural fields, under a limited range of conditions. These systems demonstrate the promise of robotic weed control technology for reducing the hand labor or pesticide application requirements of existing weed control methods (Slaughter et al., 2008). Commercial equipment is already available for non-selective patch spraying, such as the Crop Scouting Drones Miniature UAV helicopter, equipped with a camera and GPS navigation system for low-altitude aerial imaging (http://www. mikrokopter.de).

Biological control

Biological control of weeds (BCW) is defined as the action of parasites, predators, or pathogens in maintaining another organisms' population at a lower average density than the one which would occur in their absence (McFadyen, 1998). Biological control is properly employed as one of many weed management practices. It is likely that biological control of weeds will become more important than other control techniques, but it will never be the solution for all weed problems in intensive crop production. Some of the benefits of BCW are that it is: reasonably permanent, self-perpetuating, there are no additional inputs required once the agent has established itself successfully, there are no harmful side effects, the "attack" is limited to the target weed and few of its close relatives, the risks are known and evaluated before the release, control is often dependent on the host density, the spread to suitable host habitats is

self-dispersing, the costs are non-renewing, it brings high benefits (Suckling, 2013). However, BCW also has some risks such as: slow weed control, there is no guarantee of results, the establishment may fail for many reasons, there may be unknown ecological effects, some risks may not be known and cannot be evaluated in advance, it does not work well in short-term cropping cycles, the restriction of spread from the area of its initial dispersal is impossible, the initial cost, in terms of time, money and personnel needed, can be very high and weed eradication is not possible (Sheppard et al., 2003; Simberloff, 2011). The commercial applications of biological control have mainly been developed in fruit and protected cropping systems. The available systems are currently too costly and not effective enough for their use in arable crops (row crops, small grain crops, legumes, etc.). However, the establishment of wildlife features, such as beetle banks and conservation headlands, may supply organisms which would feed on the field weed species. The first classical biological control agent release against an invasive alien plant in Europe was the release of *Aphalara itadori*. Like its host, *Fallopia japonica*, *A. itadori* originates from Japan, where it is one of more than 180 insects that feed on this plant. Therefore, *A. itadori* has potentially become the first classical biological weed control agent for the European Union (Djeddour & Shaw, 2010).

Herbicide-Resistant Weeds

Repeated exposure of a weed population to any herbicide in isolation may have two effects: (1) weed species that are not controlled by the herbicide will dominate the population (species shift), and (2) the pressure will be exerted on the population to select any resistant individuals that may be present (herbicide resistance). The development of both the species shift and herbicide resistance can be effectively managed by the practice of IWM (Beckie, 2014). The implementation of IWM to avoid both of these problems considers two key aspects: (1) diversifying weed management practices and using multiple herbicide mechanisms of action (MOAs), and (2) educating the farmers about MOAs and making them aware that the discovery of new herbicide chemicals is rare, and that the indiscriminate herbicide use leads to the rapid evolution of herbicide-resistant weeds, which in turn may result in the loss of herbicide options for all weeds. Therefore, herbicide resistance management encompasses the following practices (Friesen et al., 2000; Bozic et al., 2015):

- Use of herbicide mixtures, sequences of herbicides and the rotation of herbicides that have different MOAs;
- Use of full recommended rates of herbicides, applied at the right time;
- Use of short residual herbicides whenever possible. Use of long term residual herbicides wisely and not continuously on the same field;
- Practicing crop rotations to keep any one weed species from dominating;
- Utilising tillage where applicable as a component of the weed management;
- Utilising cultural practices, reducing row spacing, maximising the crop competitiveness;
- Scouting the fields and monitoring them for resistance and weed shifts; and
- Practicing good sanitation practices to prevent the movement of weed seeds with the soil, machinery, crop residue, etc.

IWM in Herbicide-Tolerant Crops

Herbicide tolerant crops (HTC) have been developed through conventional breeding techniques (conventional herbicide tolerant crops (Miller & Al-Khatib, 2004; Bozic et al., 2012) and through gene transformation (biotech-derived herbicide tolerant crops (Reddy, 2001)). Implementing IWM for HTC is equally applicable for all types of farming systems, both in the conventional as well as in the conventional vs. biotech-derived herbicide tolerant crops. HTC currently provides many weed control benefits, such as: (1) simplified weed control, (2) better weed control, (3) reduced crop injury, (4) lower weed control costs, (5) fewer herbicide carryover problems, (6) new herbicide modes of action for the control of resistant weeds, (7) environmental benefits, (8) enabling zero tillage systems and (9) reduced fuel costs (Heap, 2012; Elezovic et al., 2012; Knezevic et al., 2013). Bearing in mind the above-mentioned benefits of growing HTC, farmers must practice diversified IWM in HTC.

Future Research Opportunities on IWM

Further research on IWM must continue to further advance the principles of weed science. Every effort must be made to move from a descriptive to a predictive science, in order to overcome the acceptance barriers. Opportunities will arise to further explore the ways to reduce management risks and the environmental impact of our agricultural production systems. Also, the agro-industry, farmers, and governments must view IWM as an important component of herbicide and environmental stewardship. Additionally, IWM is a flexible approach that is not based on prescription, however, weed scientists

must bear in mind the fact that increasing farm sizes demands simple, effective and flexible methods for weed management (Buhler, 2002). A key role for weed scientists is, therefore, to integrate the complexities of IWM into user-friendly decision support systems to meet these demands.

Ultimately, future decision support systems should incorporate different weed management strategies, past informations from the field, and real-time environmental conditions to recommend the most appropriate weed management strategies (Swanton et al., 2008). Such systems would help satisfy the growing needs for simple, effective and flexible weed management, and at the same time promote IWM practices.

CONCLUSION

This higher level of complexity partly explains why IWM has not received the same attention as integrated management of other pests. Adding to the complexity is the fact that most non-chemical tools are not as effective as herbicides, i.e. they cannot be considered as stand-alone methods, but has to be combined with other methods in a systematic way to provide sustainable and reliable weed control ("many little hammers"). Finally, the challenge for weed scientists is to develop innovative, economical IWM systems that can be integrated into current and future cropping systems to bring a more diverse and integrated approach to weed management. Because of the diversity and flexibility of weed communities, weed management needs to be a continuous process.

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INTEGRATED MANAGEMENT OF BACTERIAL DISEASES OF TOMATO AND PEPPER

Aleksa Obradovic

University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade, Serbia;
E-mail: aleksao@agrif.bg.ac.rs

ABSTRACT

Bacterial spot of tomato and pepper, caused by *Xanthomonas* spp., is a frequently occurring and widespread disease of tomato and pepper. Management of this disease currently relies on use of pathogen-free seed and transplants, elimination of volunteer plants, resistant cultivars, and frequent application of copper-based bactericides. However, these practices are ineffective in regions where hot and humid weather favor development of the disease. Novel technologies, such as application of systemic acquired resistance inducers (SAR) and use of biocontrol agents integrated with conventional practices, represent new quality in plant protection and provide increase in efficiency of the disease management.

In order to develop sustainable and integrated strategies for reducing bacterial spot severity, we investigated various combinations of biocontrol agents, including plant growth-promoting rhizobacteria (PGPR), bacterial antagonists, unformulated bacteriophages (phages) that infect the pathogen, and SAR inducers.

During three consecutive seasons, application of phages constantly provided a significant reduction in bacterial spot severity compared with the untreated control. Application of SAR-inducer compound (acibenzolar-S-methyl – ASM) significantly reduced disease severity compared to untreated control. However, integration of ASM and phage treatments provided an additional reduction in disease pressure and resulted in more efficient foliar disease control than ASM, phage, or copper-mancozeb alone.

Such approach in controlling the tomato bacterial spot pathogen was used as a model in developing similar program for pepper bacterial spot management. Host specific phage strains, isolated from substrates collected in Serbia, were used in integration with other alternative or standard treatments, and resulted in improved efficacy of the disease control strategy.

Key words: Bacterial spot, tomato, pepper, biocontrol agents, resistance inducers, bactericides

INTRODUCTION

A significant number of bacterial diseases are considered extremely destructive and therefore a threat to crops worldwide. Control of plant bacterial diseases is particularly challenging due to pathogen variability, mutation or gene transfer in the pathogen when confronted with resistance genes or bactericides, high bacterial multiplication rate during optimal conditions for disease development, and lack of adequate chemical-based control measures. Integrated disease management offers solutions for some of these challenges.

Xanthomonas spp. frequently affect tomato and pepper crops worldwide, causing bacterial spot disease. Under optimal conditions the bacterium causes leaf spotting and defoliation with reduction in yield. Due to occurrence of scabby lesions fruit marketability can be affected as well. The disease management currently relies on use of pathogen-free seed and transplants, elimination of volunteer plants, resistant cultivars,

and frequent application of copper-based bactericides. In regions with hot and humid weather favoring development of the disease effectiveness of these practices is limited. Therefore, novel technologies integrated with conventional practices, able to provide increase in efficiency of the disease management, are studied.

Bacterial diseases of tomato and pepper

There are several bacterial pathogens affecting tomato and pepper, causing widespread diseases such as bacterial spot, speck, canker, and wilt (Jones et al., 1991a). Bacterial spot of tomato and pepper, caused by *Xanthomonas* spp., is a frequently occurring disease in high temperature and high humidity conditions conducive for the disease development. Therefore, it is hard to control especially in subtropical and tropical regions with perennial occurrence of the pathogen.

Bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, is also distributed worldwide. The disease is favored by temperatures ranging from18-24°C and high humidity. All above ground parts can be affected by dark brown to black lesions which reduces fruit marketability.

Unlike bacterial spot and speck, occurrence of tomato bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) is sporadic but still can be devastating. The principal symptoms are systemic wilt of the plant and characteristic spots referred to as "bird's-eye" spots on infected fruits. It affects both field and greenhouse tomatoes originating from infected seed or transplants.

Bacterial wilt is another serious disease of tomatoes grown in regions where the pathogen *Ralstonia solanacearum* survives climatic conditions. This bacterium causes rapid death of plants with losses reaching more than 50% of the crop in naturally infested fields.

Tomato and pepper bacterial spot

Xanthomonas spp. has been shown to survive on infected tomato and pepper seed for years, in crop residue for weeks to months depending on the weather conditions, on volunteer tomato and pepper plants for at least one season after the original crop, and in association with weeds for short periods of time (Jones et al., 1986). Some studies revealed that the solanaceous weed species were unlikely hosts for the bacterium, but they may serve as short term inoculum sources (Laub and Stall, 1967).

The pathogen enters through natural openings including stomates and hydathodes and through wounds created by various factors. Infection is favored by high relative humidity and temperatures between 25° and 28°C. Under optimal conditions it causes leaf spotting and severe defoliation with great reduction in yield. Fruit marketability can be seriously affected due to occurrence of scabby lesions.

Integrated approach to bacterial spot management

Control of plant diseases is achieved by utilizing numerous regulatory, cultural, biological and chemical tactics. However, disease control still remains a challenge for farmers and crop protection specialists. In contrast to the search for efficient antifungal natural products and analogues, the task to find bactericides suitable for crop protection turned out to be exceedingly difficult. The lack of a bactericidal "silver bullet", comparable to synthetic fungicides in control of plant pathogenic fungi, has continually forced plant pathologists to search for non-pesticide control/management strategies for controlling plant bacterial diseases.

In spite of technological advances, there is no bactericide that can be efficiently used for control of plant bacterial diseases. Chemical control of tomato and pepper bacterial spot mostly relies on the application of copper-based compounds and/or antibiotics. The most frequently used antibiotics against plant bacterial diseases were formulations of streptomycin or streptomycin and oxytetracycline (McManus et al., 2002). However, resistant strains of bacterial spot pathogen emerged soon after wide-spread application of antibiotic-based compounds in plant protection (Minsavage et al., 1990; Ritchie & Ditapongpitch, 1991; Thayer & Stall, 1961). In addition, there has been a major concern in many countries that antibiotic application in the environment might cause natural resistance in many bacterial species, rendering not only these but related antibiotics useless for medical treatment.

Copper-containing bactericides proved to be an effective preventive treatment against many bacterial diseases, mostly leaf spots and blights. However, efficacy of copper bactericides for control of tomato and pepper bacterial spot was compromised by reduced sensitivity of the bacterium as a result of the excessive application of these chemicals. Copper-tolerant strains became quite prevalent in the 1980s (Jones et al., 1991b; Marco & Stall, 1983; Martin et al., 2004; Ritchie & Ditapongpitch, 1991).

Recently, a novel class of chemicals called "plant activators" has been introduced in disease management programs (Louws et al., 2001; Qui et al., 1997). One of them, acibenzolar-*S*-methyl (Syngenta Crop Protection), showed excellent potential for control of bacterial spot of tomato (Louws et al., 2001; Obradovic et al., 2004a,

2005). However, some results showed adverse effects of this compound on tomato growth and yield (Louws et al., 2001).

However, foliar applications of ammonium lignosulfonate derived from the wood pulping process, and the fertilizer potassium phosphate, neem oil and fish emulsion, compost water extracts, were tested for their ability to control bacterial spot under both greenhouse and field conditions (Abbasi et al., 2002, 2003; Al-Dahmani et al., 2003).

Among the limited number of biological agents commercially available for the control of bacterial diseases, the most encouraging results were obtained using host specific phages for control of bacterial spot on tomato (Balogh et al., 2003; Flaherty et al., 2000; Jones et al., 2014; Obradovic et al., 2004a).

However, none of the above non-chemical treatments have been able to stand alone and provide efficient control. A combination of practices, such as the use of pathogen-free seed and transplants, elimination of volunteer plants, resistant tomato and pepper cultivars, and frequent application of a copper and mancozeb mixture (Momol & Pernezny, 2005) reduces the damage from bacterial spot. However, there is a permanent need to develop more effective and sustainable disease management strategies. Therefore, the integration of various combinations of biocontrol agents, including plant growth-promoting rhizobacteria (PGPR), bacterial antagonists, unformulated and formulated bacteriophages (phages) specific to the pathogen, and systemic acquired resistance inducers (SAR) was investigated.

During three consecutive seasons, application of phages constantly provided a significant reduction in bacterial spot severity compared with the untreated control. In addition, phage-treated plants produced significantly more marketable fruits than plants not receiving the phages. Although copper-sensitive strains were used in this study, which favored more effective control of bacterial spot with copper bactericides, phages applied twice a week were either more effective or equally effective compared with the standard copper-mancozeb treatment. Application of SAR-inducer significantly reduced disease severity compared to untreated control. However, integration of ASM and phage treatments provided an additional reduction in disease pressure and resulted in more efficient foliar disease control than ASM, phage, or copper-mancozeb alone. Both phages and ASM have unique modes of action, targeting the pathogen only, not affecting beneficial microbiota and not overlapping with any of conventional practices that are in use at present. Being compatible with other treatments, they are suitable for integration in complex tomato disease and pest management programs (Momol et al., 2002).

Similar approach in tomato bacterial spot control was used as a model in developing program for pepper bacterial spot management. Host specific phage strains, isolated from substrates collected in Serbia, were used in combination with other alternative or standard treatments, and resulted in improved efficacy of the disease control strategy. An integration of the phage application with standard copper treatments, resistant cultivars, or use of plant resistance activators in susceptible cultivars, reduced pepper bacterial spot severity in conditions of artificial inoculation favoring the pathogen (Gašić et al., 2014; Šević et al., 2014).

CONCLUSIONS

- Plant protection from bacterial diseases is a complex strategy that includes beneficial effects of many factors contributing to the efficacy and sustainability of the disease management and production of safe food.
- Both phages and ASM have unique modes of action, targeting the pathogen only, not affecting beneficial microbiota and not overlapping with any of conventional practices that are in use at present. Being compatible with other treatments, they are suitable for integration in complex tomato disease and pest management programs.
- Integrated with other cultural practices (disease free seed, resistant cultivars, crop rotation...) these alternative treatments could result in control of tomato and pepper bacterial spot and reduce its impact to tolerable level.

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INTEGRATED PROTECTION OF FIELD CROPS, VEGETABLES AND STORED PRODUCTS

ИНТЕГРИРОВАННАЯ ЗАЩИТА ПОЛЕВЫХ И ОВОЩНЫХ РАСТЕНИЙ И ПРОДУКТОВ В СКЛАДСКИХ ПОМЕЩЕНИЯ

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INTEGRATION OF BIOLOGICAL AND CHEMICAL METHODS IN CONTROL OF PEPPER BACTERIAL SPOT

Milan Šević[1], Katarina Gašić[2], Mladen Đorđević[1], Maja Ignjatov[3], Mirjana Mijatović[1] Bogoljub Zečević[1] and Aleksa Obradović[4]

[1]Institute of Vegetable Crops, Karađorđeva 71, Smederevska Palanka, Serbia
[2]Institute for Plant Protection and Environment, Teodora Drajzera 9, 11040 Belgrade, Serbia
[3]Institute of Field and Vegetable crops, Maksima Gorkog 30, Novi Sad, Serbia
[4]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade – Zemun, Serbia
sevicmilan@yahoo.com

ABSTRACT

Bacterial spot, caused by *Xanthomonas euvesicatoria*, is widely spread disease of pepper in Serbia. When weather conditions are favorable for disease development, pepper producers do not have adequate resources to control this pathogen. Copper based bactericides registered in our country are not effective enough. However, effective protection could be achieved only by integrating positive effects of different protection methods. In order to develop sustainable and integrated control strategy for this disease, we investigated various combinations of biological control agents and chemicals. Intensity of the disease ranged from 31 to 59% on untreated control plants. All integrated treatments were effective against *X. euvesicatoria* and significantly reduced disease severity in all experiments, compared to untreated control. The most efficient treatment was integration of acibenzolar-S-methyl, copper hydroxide and bacteriophages, reducing disease severity 97-99%. This combination may be an effective new tool for pepper growers to manage bacterial spot.

Keywords: *Xanthomonas euvesicatoria*, copper compounds, acibenzolar-S-methyl, antagonist, bacteriophage

INTRODUCTION

Bacterial spot, caused by *Xanthomonas euvesicatoria* (Jones et al., 2004) is the most important pepper disease in Serbia. The intensity of infection and economic losses depend on the cultivar susceptibility, applied protection measures and weather conditions. Routine disease management practices, such as use of good quality seed, crop rotation, growth of less susceptible cultivars and application of copper compounds, have failed to provide satisfactory disease control, especially when weather conditions favored the spread of the pathogen. Application of copper compounds alone proved less effective than in combination with ethylene-bis-dithiocarbamates (EBDC) fungicides (Marco et al., 1983). However, due to the frequent application, copper tolerant or resistant strains of *X. euvesicatoria* were reported (Marco et al., 1983; Adaskaveg et al., 1985). The use of antibiotics, especially streptomycin, for bacterial diseases control has begun in the 50's. Successful control of *X. euvesicatoria* in tomato and pepper crops did not last long due to streptomycin resistant bacteria populations observed in the early sixties (Stall and Thayer 1962). Streptomycin-resistant strains spread rapidly and became widely distributed (Argentina, Brazil, California, Florida, Georgia, Ohio, Pennsylvania and Taiwan), forcing plant pathologists to search for other solutions (Obradović et al., 2004).

According to the recent literature data, biological agents as bacteriophages and some new alternative

methods such as resistance inducers and harpin protein represent new strategies in control of *X. euvesicatoria* in tomato crops (Obradović et al., 2004, 2005; Jones et al., 2007). Treatments of acibenzolar-S-methyl in combination with bacteriophages, or bacteriophages and harpin protein, significantly reduced bacterial spot of tomato in the fields of Florida (Obradović at al., 2004).

The aim of our research was to study efficacy of various combinations of biological control agents and chemicals in order to develop sustainable and integrated control strategy for bacterial spot control.

MATERIAL AND METHOD

The experiments have been conducted on the experimental field of the Institute of Vegetable Crops in Smederevska Palanka. In order to develop sustainable and integrated control strategy for pepper bacterial spot, we investigated various combinations of biological control agents: bacteriophages (strain КΦ-1(Gašić at al., 2011), conc. $2.3x10^{1o}$ PFU/ml) and *Bacillus subtilis* (strain AAac, conc. 10^8 CFU/ml and Serenade AgraQuest, Inc conc. 0.4%), systemic acquired resistance inducer acibenzolar-S-methyl (ASM, Bion 50WG, Syngenta crop protection; 0.003%) and copper hydroxide (Kocide 2000, DuPont, 0.19%) (Table 1). Copper hydroxide was applied as a standard treatment one day before inoculation and then once a week. *Bacillus subtilis* treatment was applied one day before inoculation and then once a week. ASM was applied 9 and 4 days prior to inoculation and after that at 14 day intervals. The total number of three aforementioned treatments was six. Nonformulated bacteriophages were applied immediately prior to inoculation followed by twice a week at dusk, with a total of 12 treatments. Pepper plants (cv. Early California Wonder) were artificially inoculated 9 days after transplanting. Inoculation was done by spraying water suspension of *X. euvesicatoria* strain KFB 13 (sensitive to copper compounds) (conc. 10^8 CFU/ml) using hand-held mister. Concentration of bacteria in the suspension was adjusted to 10^8 CFU/ml using McFarland's scale and confirmed by a serial dilution plating method (Klement et al., 1990). Noninoculated and inoculated tap water-treated plants were used as a control. The experiment was repeated two times. Each treatment consisted of four replications and the experiment was designed as a complete randomized block system. Percentage of the leaf surface covered with necrotic spots was evaluated by using the Horsfall-Barratt (HB) rating scale, 7 days after application of the last treatment (Horsfall-Barratt, 1945). Data were analyzed by applying one-way ANOVA and Duncan's multiple range test.

RESULTS AND DISCUSSION

Results of two field experiments showed that all integrated treatments were effective against *X. euvesicatoria* and significantly reduced disease severity in all experiments, compared to untreated control (Table 1). Intensity of the disease ranged from 31 to 59% on untreated control plants. The most efficient treatment was integration of acibenzolar-S-methyl, copper hydroxide and bacteriophages, reducing disease

Table 1. Efficacy of integration of biological and chemical methods in control of pepper bacterial spot.

Treatments	Concentration	Experiment 1		Experiment 2	
		Mean *	Efficacy %	Mean*	Efficacy %
Bion 50 WG + Kocide 2000 + Bacteriophage	0.003% + 0.19% + $2.3x10^{10}$ PFU/ml	1.463 E	97.5	0.293 B	99.0
Bion 50 WG + Kocide 2000	0.003% + 0.19%	2.925 DE	95.0	1.463 B	95.3
Kocide 2000 + Bacteriophage	0.19% + $2.3x10^{10}$ PFU/m	3.511 DE	94.0	2.633 B	91.5
Kocide 2000 + Bacteriophage + Serenade	0.19% + $2.3x10^{10}$ PFU/m + 0.4%	5.268 CDE	91.1	1.755 B	94.3
Kocide 2000	0.19%	6.439 CDE	89.1	4.095 B	86.8
Kocide 2000 + Serenade	0.19 + 0.4%	8.198 CDE	86.1	4.389 B	85.9
Bion 50 WG + Serenade + Bacteriophage	0.003% + 0.4% + $2.3x10^{10}$ PFU/ml	9.370 CDE	84.2	2.633 B	91.5
Bion 50 WG + Bacteriophage	0.003% + $2.3x10^{10}$ PFU/ml	11.714 CD	80.2	2.048 B	93.4
Bion 50 WG + Antagonist (Strain AAac)	0.003% + 10^8 CFU/ml	13.474 BC	77.3	4.096 B	86.8
Bion 50 WG + Serenade	0.003% + 0.4%	20.506 B	65.4	2.340 B	92.5
Untreated inoculated control	-	59.375 A	-	31.250 A	-

*Means followed by different letters within a column are significantly different according to Duncan's multiple range test, P = 0.05 level.

severity 97-99%. However, in experiment 2 there was no statistically significant difference in efficacy of standard treatment (copper hydroxide) and various integration of biological control agents: bacteriophages *and Bacillus subtilis* strains and ASM. ASM alone was not effective for controlling bacterial spot in the field as was observed in greenhouse and climatic chamber conditions (Šević at al., 2011, 2011a, 2012). Biweekly applications of ASM and applications of bacteriophages twice a week at dusk significantly reduced the disease severity (80-93%). In these experiments we used non-formulated bacteriophages. Balogh et al. (2003) reported that formulation of bacteriophages with skim milk and sucrose contributed to greater stability on leaf surfaces and therefore better efficiency. In the present study copper and antibiotics-sensitive strain of the pathogen was used, favoring more effective disease control with standard copper hydroxide (86-89%). However, these integrated treatments may be relatively more effective compared to the standard when copper resistant *X. euvesicatoria* strains are predominant in natural epidemics. In this study we demonstrated that some alternative methods (bacteriophages, ASM) could serve as a new promising tool for pepper producers to control bacterial spot.

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EFFECT OF THE COMBINED APPLICATION OF A LOW-FREQUENCY PULSE ELECTRIC FIELD AND QUADRIS AND IZABION PREPARATIONS ON THE DISEASE PROTECTION AND YIELD INCREASE OF POTATO

Maria Kuznetsova, Natalia Statsyuk, Alexander Rogozhin, Tatiana Smetanina and Alexey Filippov

All-Russian Research Institute of Phytopathology, Moscow region, 143050, Russia, e-mail: mari.kuznetsova@gmail.com

SUMMARY

The effect of a new integrated system on the disease protection and yield increase in potato was studied. The proposed scheme includes the pre-planting treatment of seed tubers with modulated low-frequency pulse electric field (EF), in-furrow application of the azoxystrobin-containing Quadris fungicide during the planting, and the use of the Izabion biofertilizer, containing 62.5% of amino acids and peptides, in the tank mix with standard fungicides for the routine spraying of plants. The trials included six experimental schemes: (1) EF treatment followed by the standard scheme of fungicidal treatments; (2) Quadris application followed by the standard scheme of treatments; (3) use of the tank mix of Izabion and fungicides according to the standard scheme of treatment; (4) standard scheme of treatment (treated control) including five fungicidal sprayings (fluazinam (Shirlan), mephenoxam+mancoceb (Ridomil Gold MC, 2x), mandipropamid+diphenoconazol (Revus+Skor), and fluazinam (Shirlan)); (5) integrated protection scheme including all mentioned treatments; (6) unprotected control (no any treatments). The following parameters were assessed: the development of rhizoctonia infection on shoots, the development of late and early blight infections on vegetating plants, and the development of rhizoctonia and silver scurf infections on daughter tubers. In addition, the germination level of mother tubers was observed.

The proposed integrated scheme significantly reduced the harmfullness of all studied plant diseases. Comparing to the treated control, the tested experimental scheme increased the yield and the marketable fraction of potato tubers by 8.5 t/ha and 33%, respectively; comparing to the unprotected control, the total increase made 14.7 t/ha and 53%, respectively, that makes it to be very promising for the potato-growing industry.

Key words: *Phytophthora infestans*, *Alternaria solani*, *Alternaria alternata*, *Rhizoctonia solani*, *Helminthosporium solani*, integrated protection system, potato, azoxystrobin, biofertilizer, pulse electric field

INTRODUCTION

Potato is one of the most important crops in Russia, which, being inferior to China and India, takes the third place in the world potato production (FAOSTAT, 2015). At the same time, potato productivity in Russia still remains very low. One of the reasons of such situation is the yield loss caused by various pathogenic microorganisms. The most common and devastating potato diseases in Russia are late blight (*Phytophthora infestans*), early blight (*Alternaria solani* and *A. alternata)*, black scurf (*Rhizoctonia solani),* silver scurf (*Helminthosporium solani),* and black dot (*Colletotrichum coccodes*) (Anisimov et al., 2009).

The most serious yield losses in Russia are caused by the late and early blight of potato. In the case of the late blight, the resulting average annual yield losses make 4 mln. tons (Filippov, 2012). In the case of early blight

outbreaks, the corresponding yield losses can reach 40%. A common way to control these diseases is the use of fungicides. The number of chemical fungicides approved for the use against these two diseases on the territory of Russia makes several dozens (Anonymous, 2014).

In the case of black and silver scurf and black dot, the corresponding loss of potato shoots can reach 15-20%. The only preparation approved for the in-furrow use in Russia is azoxystrobin-based Quadris fungicide (Anisimov et al., 2009). Azoxystrobin is highly soluble in water ($\log_{Pow}$ = 2.64), so its molecules are able to easily penetrate into plant tissues; after their active uptake by roots, they are acropetally transferred to plant stems and leaves (Gisi, 2002).

The harmfulness of the above-mentioned diseases can be significantly reduced by the use of integrated protection systems (IPS) providing an increased yield, better product quality and additional economical and environmental advantages connected with the reduction of the number of fungicidal treatments. Along with the use of modern fungicides, such systems can include the use of healthy seed material, resistant potato cultivars, proper land treatment, and other activities able to improve plant resistance (Kuznetsova, 2007).

A new Izabion BP preparation, recently registered in Russia, represents the last-generation organic fertilizer suitable for many agricultural crops, including potato. This preparation represents a source of readily assimilable amino acids and peptides, is characterized by a rapid uptake and transportation into plant tissues, and has an excellent compatibility with almost all pesticides, excepting copper-based ones (Kuznetsova et al., 2012). Being environmentally friendly, Izabion BP seems to be very promising for the use in various IPS.

Electrophysical treatment of seed potato is another potential environmentally friendly component of IPS. Electrical and magnetic fields provide a significant influence on the morphological, physiological, and biochemical characteristics of plants. Many publications and patents describe the positive influence of electromagnetic fields on the seed germination rate, plant development, and the resulting crop capacity in various crops (Moon and Chung, 2000; Cramariuc et al., 2005; Costanzo, 2008; Molamofrad et al., 2013). However, there is a small number of publications, devoted to the possible influence of electromagnetic treatment on the disease resistance of plants, though this direction of studies seems to be very promising. After a long-term research program, our lab developed and patented the method of treatment of seed material with a specially modulated low-frequency pulse electric field (EF), which increases the yield and improves the resistance of various agricultural crops to some diseases, including the late blight of potato (Kuznetsova et al., 2000; Bel'kovets et al., 2012).

In this study we assessed the effect of a new integrated protection system including physical, chemical and biological treatments on the disease resistance and yield increase in potato. The offered scheme includes the pre-planting EF-treatment of seed tubers, in-furrow application of the azoxystrobin-containing fungicide (Quadris) during the planting, and the use of the Izabion biofertilizer in the tank mix with standard fungicides for the routine spraying of plants.

MATERIALS AND METHODS

Trial arrangement

Small-plot field trials were arranged on the experimental field of the All-Russian Research Institute of Phytopathology (Moscow region). Five different variants of treatment were tested against unprotected control (Table 1). The area of each experimental plot was 25 m^2; the plots were randomly located on the field. Each variant was tested in four replications.

Table 1. Arrangement of the experiment on the assessment of the effect of integrated application of the low-frequency pulse electric field (EF), azoxystrobin-containing Quadris fungicide, Izabion biofertilizer, and routine fungicides on potato

Scheme of protection	Pre-planting EF treatment (EF)	In-furrow Quadris application during the planting[1] (Q)	Treatment during the vegetation season	
			Izabion application[2] (I)	Routine fungicidal treatment[3] (R)
EF+R	+	-	-	+
Q+R	-	+	-	+
I+R	-	-	+	+
R	-	-	-	+
IPS	+	+	+	+
Control	-	-	-	-

[1]Azoxystrobin-containing Quadris fungicide (3 l/hectare).
[2]Izabion biofertilizer containing 62.5% of amino acids and peptides (2 l/hectare in a tank mix with routine fungicides).
[3]Standard highly-efficient scheme of fungicidal treatments (protected control): (1) fluazinam (Shirlan), (2) mephenoxam + mancoceb (Ridomil Gold MC, 2x treatment), (3) mandipropamid + diphenoconazol (Revus+Skor), and (4) fluazinam (Shirlan).

Potato (cv. Red Scarlett) was planted on May 13 and harvested on September 8. The land treatment of the field included under-winter ploughing; spring ploughing; pre-planting furrow formation; application of organic fertilizers for the precursor crop (35 tons/hectare); pre-planting application of inorganic fertilizers (40 kg of active substance per a hectare); and a pre-emergence treatment with a Zenkor herbicide (1 kg/hectare).

Electric field treatment

Three days before planting, potato tubers were treated for 18 h with a modulated low-frequency pulse electric field electric field (EF). The detailed description of basic field parameters is given in the corresponding patent (Bel'kovets et al., 2012).

Biofertilizer application

Izabion biofertilizer, containing 62.5% of amino acids and peptides as active ingredients, was applied during the vegetation season (2 l/hectare) in a tank mix with standard fungicides used for a routine spraying of plants.

Assessment methods

The following parameters were assessed in the course of the study: the development of rhizoctonia infection on shoots, the development of late and early blight infections on vegetating plants, and the development of rhizoctonia and silver scurf infections on daughter tubers. In addition, the germination level of mother tubers and the total yield were determined. In the case of the rhizoctonia disease and silver scub, the assessment of the disease severity was carried out according to the EPPO standards (EPPO, 1997). In the case of the late and early blight of potato, the severity of disease was determined according to the British Mycological Society scale (James et al., 1972).

RESULTS AND DISCUSSION

Results of the tuber germination assessment are shown on Fig. 1. The treatment with either routine fungicides or their combination with Izabion did not influence on the percentage of germinated tubers as compared with the control variant, whereas other three variants significantly increased it with the maximum value (95%) for the proposed IPS.

The results of assessment of the rhizoctonia disease severity are shown in Table 2. The injuriousness of the rhizoctonia infection was reduced in the case of protection schemes, which included the combination of routine treatment with the EF treatment or Quadris application, and also for the IPS.

The results of the assessment of a silver scurf infection level for different protection schemes are shown in Fig. 2. Only schemes, which include in-furrow azoxystrobin application, showed a significant reduction of the infection level.

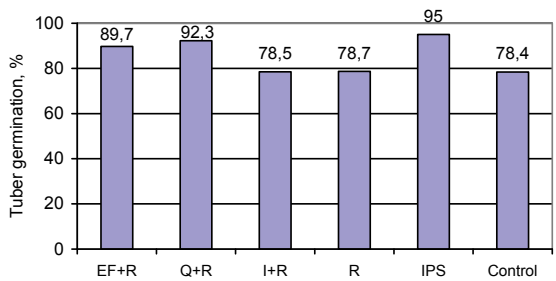

Figure 1. Germination rate of seed potato tubers determined for the tested protection schemes. *EF*, electrical field treatment; *Q*, Quadris application; *I*, Izabion application; *R*, routine treatment; *IPS*, integrated protection system. $LSD_{0.90}$ = 3.7.

Table 2. Severity of rhizoctonia infection on potato determined for the tested protection schemes

Variant	Average number of stems per a plant	Average number of infected stems per a plant	Average stem infection level, scores	Average stolon infection level, scores
EF+R	3,9	0,4[1]/1,2[2]	0,5/1,0	Single / 0,9
Q+R	4,0	0 / 0,2	0 / single[3]	0 / single
I+R	3,7	1,0/1,7	1,0/0,9	1,1/0,9
R	3,5	1 / 1,8	1 / 1,0	1,4 / 1,0
IPS	4,2	0 / 0,2	0 / single	0 / single
Control	3,6	1 / 1,9	1 / 1,0	1,3 / 1,0
$LSD_{0,90}$	0,6	-/ 0,4	-	-

[1] The observation was made at the full sprouting phase.
[2] The observation was made 13 days prior the harvesting.
[3] Only single manifestations of infection were observed.

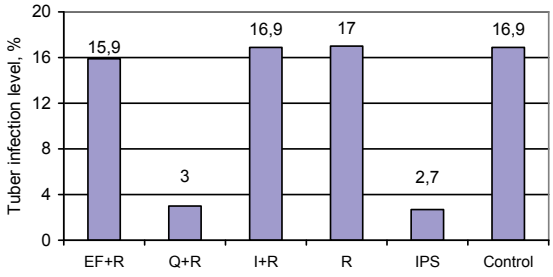

Figure 2. Level of a daughter tuber infection with silver scurf in the compared schemes of protection. *EF*, electrical field treatment; *Q*, Quadris application; *I*, Izabion application; *R*, routine treatment; *IPS*, integrated protection system. $LSD_{0.90} = 3.7$.

The level of the total late and early blight infection, determined for the tested schemes, is shown in Fig. 3. All tested schemes significantly delayed the primary manifestations of the infection as compared with the untreated control. To the end of the observation period, the level of infection in the untreated control reached 90% against 9% in the treated control (R variant). The same values for other tested variants varied from 5 (I+R) to 8 (Q+R) and 18% (EF+R), and only in the case of IPS the final infection level still remained within 1%.

The results of the assessment of the total yield and the marketable fraction of tubers are shown in Fig. 4. All variants tested significantly exceeded the control in both parameters. A significant difference in the total yield was also revealed in EF+R, Q+R, and I+R variants comparing the treated control (R variant); in the case of the marketable fraction, a significant difference against the treated control was observed in EF+R and Q+R variants. Again, the IPS variant showed the best values reaching 350 centner/hectare of the total yield and 95% of the marketable fraction of tubers.

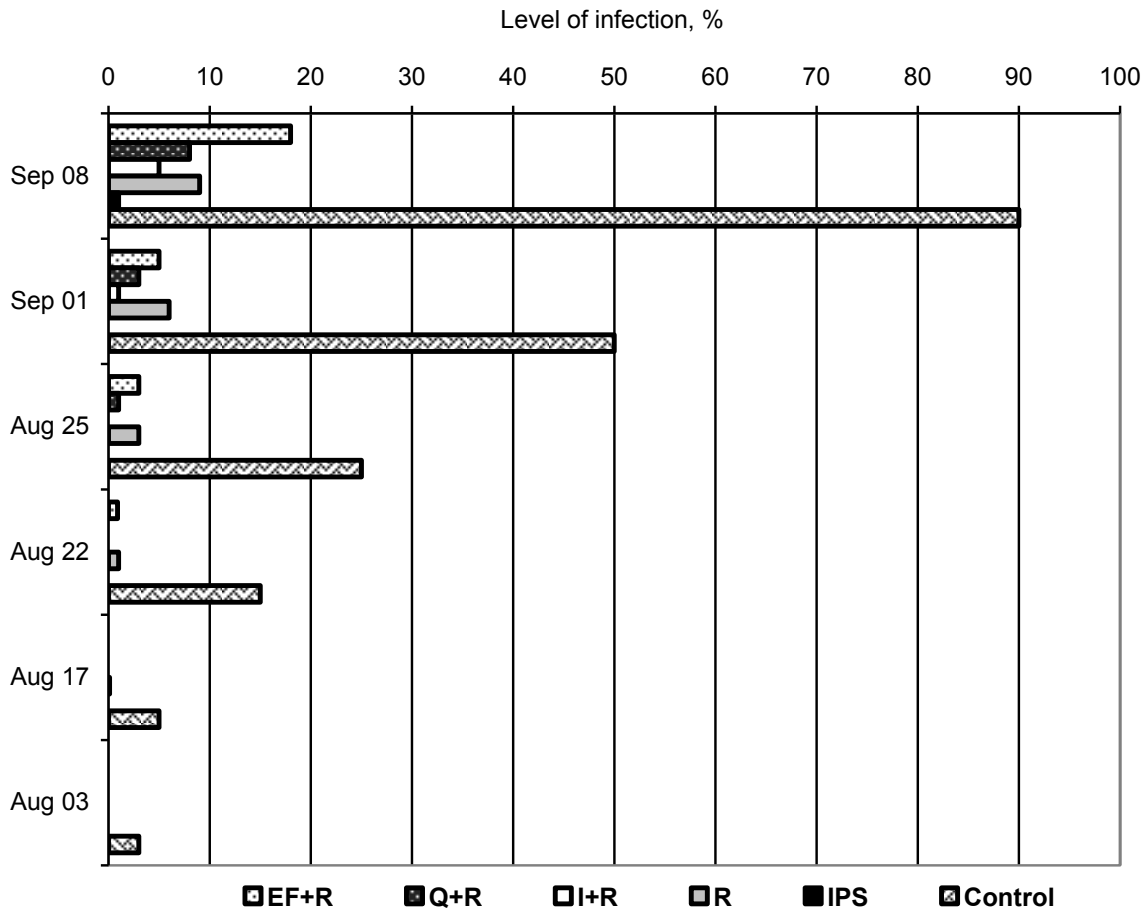

Figure 3. Level of the total late and early blight infection of potato plants determined for the compared schemes of protection. *EF*, electrical field treatment; *Q*, Quadris application; *I*, Izabion application; *R*, routine treatment; *IPS*, integrated protection system. $LSD_{0.90} = 3.7$.

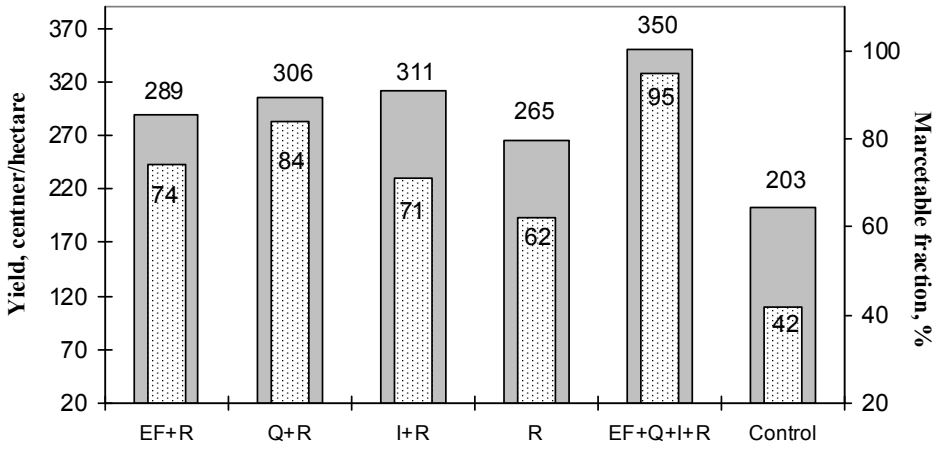

Figure 4. Total potato yield ($LSD_{0.90}$ = 25.3) and the marketable fraction of tubers ($LSD_{0.90}$ = 10.2) in the compared schemes of protection. *EF*, electrical field treatment; *Q*, Quadris application; *I*, Izabion application; *R*, routine treatment; *IPS*, integrated protection system

Summarizing all obtained results, one can conclude the following. The application of the EF+R scheme increased the tuber germination by 11.3%, reduced the severity of a rhizoctonia disease, and delayed the primary manifestation of the late blight for one week; the yield increase made 2.4 t/ha comparing to the treated control (R). The Q+R scheme increased the germination by 13.9%, reduced the severity of rhizoctonia infection, delayed the primary manifestation of the late and early blight, and suppressed a silver scurf infection on daughter tubers. The yield increase for this variant was 4.1 t/ha comparing to the treated control (R). The I+R scheme reduced the late and early blight development and increased the yield by 4.6 t/ha comparing to the treated control. Finally, the IPS scheme significantly reduced the severity of all studied plant diseases. Comparing to the treated control (R), this scheme increased the yield and the marketable fraction of potato tubers by 8.5 t/ha and 33%, respectively; comparing to the unprotected control, the total increase made 14.7 t/ha and 53%, respectively.

The decision to study the effect of the azoxystrobin-containing Quadris fungicide on the late blight of potato was made due to the fact that in recent years we observed very early manifestation of this disease on potato fields of Russia that required early application of fungicides. We suppose that the in-furrow Quadris application during the planting is able not only to reduce the harmfulness of the black and silver scurf and black dot of potato, but also to delay the development of the late and early blight on potato plants.

In the case of the Izabion, earlier we revealed that the treatment of potato plants with the mix of this biofertilizer with fungicides significantly reduces the level of infection with the late and early blight as compared with the use of fungicides without Izabion and increases the total yield (Kuznetsova et al., 2012). Moreover, it is known that foliar fertilizers represent the fastest way to eliminate the deficiency of nutrients in plants (Ryabtseva et al., 2005).

Finally, the decision to examine the possibility to include the pre-planting EF treatment of tubers into the integrated protection system was made on the basis of our earlier studies, which demonstrated a double reduction of the infection ability of *P. infestans* conidia after their EF treatment and the prolonged suppression of the late blight manifestation on leaves of potato plants. In addition, a significant reduction of the fraction of infected daughter tubers was observed (Kuznetsova, 2000). In addition, this treatment technology is very simple, environmentally friendly, and provides a possibility of a simultaneous treatment of large volumes of seed material, i.e. is suitable for a large-scale use.

Thus, the offered system of integrated potato protection, which includes the use of pre-planting treatment of seed tubers with modulated electrical field, in-furrow application of the azoxystrobin-containing Quadris fungicide (3 l/hectare), and the use of a tank mix of a biofertilizer Izabion (2 l/hectare) with fungicides used for routine protective treatments of plants, significantly reduces the development of the studied potato diseases and increases both the total yield and the marketable fraction of tubers that makes it to be very promising for the potato-growing industry.

CONCLUSIONS

The proposed integrated protection scheme significantly delays the development of the studied potato diseases and reduces their severity. Comparing to the routine scheme of fungicidal treatment, this scheme increased the yield and the marketable fraction of potato tubers by 85 centner/ha and 33%, respectively; comparing to the unprotected control, the total increase made 147 centner/ha and 53%, respectively. Thus, the integrated scheme of potato protection demonstrates excellent results that makes it to be very promising for the use in the potato-growing industry.

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CHEMICAL AND BIOLOGICAL CONTROL OF CULTIVATED MUSHROOM DISEASES

Ivana Potočnik*, Emil Rekanović, Miloš Stepanović, Svetlana Milijašević-Marčić and Biljana Todorović

Institute of Pesticides and Environmental Protection, Banatska 31B, P.O. Box 163, 11080, Belgrade-Zemun, Serbia; E-mail: ivana.potocnik@pesting.org.rs.*

ABSTRACT

Button mushroom (*Agaricus bisporus*) production worldwide and in Serbia has been expanded over the past decade. Introduction and spread of many fungal and bacterial pathogens results in lower yield and quality. Until recently, *Mycogone perniciosa, Lecanicillium fungicola*, and *Cladobotryum* sp., the causal agents of dry bubble, wet bubble, and cobweb disease, respectively, had been considered the main *A. bisporus* pathogens. Besides these pathogenic fungi, mushroom virus X and various *Trichoderma* species, the causal agents of green mould, have also emerged as major pathogens in the past decade. The most serious of them, *T. aggressivum* f. sp. *europaeum*, has been transmitted from the British Isles to many European countries including Serbia. In addition, the bacterial pathogen *Pseudomonas tolaasii* has been found to be associated with brown blotch symptoms. Only few fungicides are officially recommended in mushroom industry as a small crop: prochloraz in the EU countries, and chlorothalonil, thiabendazol and tiophanate-methyl in North America. Latterly, a decreased sensitivity of *L. fungicola* to prochloraz has been observed in Spain. Prochloraz inefficiency in cobweb disease control has also been recorded in Great Britain. As a result some new biological antimicrobial supstances are being tested. Biofungicides based on tea tree oil and *Bacillus subtilis* cause a significant reduction in cobweb and green mould disease incidences in *A. bisporus* growing rooms. The antimicrobial activity of many essential oils has been tested and oregano, thyme and mint oils were found the most efficient substances against various mycopathogens. Recently, an eco-friendly disinfectant based on colloidal silver and hydrogen peroxide has been found to suppress significantly the growth of *P. tolaasii*. Furthermore, peracetic acid has proved to be an efficient casing soil disinfectant against cobweb disease. This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project TR31043.

Key words: mushrooms, mushroom diseases, mushroom disease control.

INTRODUCTION

Button mushroom (*Agaricus bisporus*), oyster mushroom (*Pleurotus* sp.) and shiitake (*Lentinula edodes*) are the most commonly cultivated basidiomycetes worldwide, as well as in Serbia. As a result of popularization of mushroom farming, mushroom production continues to increase. World production of button mushrooms is over 3 million tonnes annually. European countries account for 50% of the overall world production, of which 87% belong to EU countries and 13% to countries outside the EU (Chang, 1999). Commercial strains of cultivated mushrooms produce considerable yield, but they are susceptible to a variety of viral, bacterial and fungal diseases. Pathogens have a significant effect on yield and quality, and mushroom production in Europe sustains an annual loss of approximately 20% (Grogan, 2008). The most important pathogens of edible mushrooms are several fungi: *Lecanicillium fungicola*, *Mycogone perniciosa,*

Cladobotryum spp. and *Trichoderma* spp., as well as the bacterium *Pseudomonas tolaasi* and viruses La France and mushroom virus X (MVX). The causal agent of dry bubble disease is *Lecanicillium* (*Verticillium*) *fungicola*. The disease has three types of symptoms: spotty cap, stipe blowout and fruiting body malformation. *Mycogone perniciosa* causes wet bubble, which results in mushroom transformation into large, irregular, nodular and tumorous fungal masses with tear-drop phenomenon of exudation of accumulated extracellular fluid. *Cladobotryum* spp. is the causal agents of cobweb disease. Disease symptoms include: cottony fluffy white or yellowish to pink colonies on mushroom casing, rapid colonization of casing surface, covering of mushroom fruiting bodies by mycelia, and their decay (Potočnik *et al*, 2008b; 2010a; 2010b). Green mould disease is charactrized by white mycelia of fast-growing colonies on casing or compost that changes colour into green after extensive sporulation. Rusty spots on mushroom fruiting bodies are early and accompanying symptoms. In serous outbreaks, infected areas produce no mushrooms and are surrounded by red-pepper mites (*Pygmephorus* spp.) feeding on the pathogenic fungi (Kosanović *et al*., 2013). Spores of *Lecanicillium*, *Mycogone* and *Trichoderma* sp. are sticky and spread by insects, personnel or equipment. Contrarily, *Cladobotryum* conidia are dry, large and aerodynamic, and spread by ventilation system to all parts of farms (Potočnik *et al*., 2010a). Brown blotch is a bacterial disease caused by *Pseudomonas tolaasii*. Colonisation of wet mushroom caps by the bacterium results in brown spots and lesions. Lesions may coalesce to cover the entire mushroom surface (Milijašević-Marčić *et al*., 2012). La France virus was probably the first major pathogen impacting severely on the mushroom industry in the 1960s. It is characterized by slow-growing mycelia and abnormal fruit bodies. It was predominantely spread by spores from infected mushrooms and also by infected mycelium. The disease was controlled by cooking out crops at the end cycle. Mushroom Virus X (MVX) is a disease that emerged in the late 1990s and its symptoms were associated with a variable number of viral double-stranded RNAs. Disease symptoms include bare cropping areas, crop delay, premature veil opening, brown-colored mushrooms and malformations (Grogan, 2008). Fourty years ago, fungal diseases reduced the production of *A. bisporus* in Europe and North America by 5%. A cobweb epidemic of the mid-1990s increased yield losses up to a significant 40%. (Fletcher *et al*., 1989). Brown bacterial blotch was responsible for losses of 10% worldwide (Milijašević-Marčić *et al*., 2012). In the more recent period, major button mushroom diseases have been caused by mushroom virus X complex and *Trichoderma* species, both accounting for losses ranging between 30% and 100% (Grogan, 2008). In the 1970s, green mold was a minor problem and caused by various *Trichoderma* species, but later it became very aggresive and spread all over the world. That new forms have designed as *T. aggressivum* f. sp. *aggressivum* in North America and *T. a*. f. sp. *europaeum* in Europe (Kosanović *et al*., 2013). Dry and wet bubbles induced great losses in mushroom production in Serbia during the mid-1990s, (Potočnik *et al*., 2008b; 2010b). Over the past ten years, cobweb disease has caused significant damage in mushroom cultivation in our region, while green mould has become the most serious problem in Serbian mushroom industry over the past few years (Potočnik *et al*., 2010a; Kosanović *et al*., 2013).

Chemical disease control of cultivated mushrooms

A common method of pathogen control on mushroom farms is treatment of casing soil by disinfectants and fungicides. Disinfectants that are regularly used in Serbia are: formalin (72%), sodium dichloroisocyanurate (5%), active oxygen (5%), sodium hypochlorite (14%), lime (23%), sulfur (14%) and potassium permanganate (9%). Calcium chloride and chlorinated compounds are the most commonly used chemicals for bacterial disease control (Milijašević-Marčić *et al*., 2012). The ongoing EU pesticide reviews have resulted in withdrawed approval for the use of many chemicals. The resulting major challenges include disease control with only a few chemicals, and fungicide resistance in pathogen populations. As studies of fungicides efficacy on cultivated mushrooms as small crops by agrochemical companies are very rare, only a few fungicides are officially recommended in mushroom industry: prochloraz in the EU countries and South Africa, and chlorothalonil, thiabendazol and tiophanate-methyl in the USA and Canada (Beyer and Kremser, 2004; Grogan, 2008). The Australian mushroom industry has three authorised fugnicides to use against fungal diseases: prochloraz, carbendazim and thiabendazole (Allan *et al*., 2008). In the late 1970s, resistant strains of *L. fungicola* to benzimidazoles occurred (Fletcher *et al*., 1989). The resistance of *Cladobotryum* spp. to benzimidazoles was reported after their extensive use in the British Isles in the 1990s (Grogan, 2008). Serbian *C. dendroides* isolates became weakly resistant to thiophanate-methyl (Potočnik *et al*., 2009a). Initially, benzimidazole fungicides applied to spawn in the US provided good control of the problem with *T. agressivum*, but resistance has emerged neverthless,

leaving improved hygiene as the only option for control of this pathogen (Beyer and Kremser, 2004). The most effective fungicide in mushroom disease control is prochloraz. Decreased sensitivity of the pathogenic fungi *Lecanicillium* and *Cladobotryum* to prochloraz has been already recorded in Spain and Great Britain (Gea *et al*., 2005; Grogan, 2008). *L. fungicola* tolerance to prochloraz has not yet been associated with any major loss, probably because of the fact that *Lecanicillium* teleomorph has not been encountered so far, reducing the risk of increased resistance due to genetic recombination. Prochloraz has been found to be degraded by microbes present in the casing soil, and its concentration drops considerably by the end of the second flush and less than 15% remaining by day 45 (Grogan, 2008). Further research has indicated that prochloraz degrades much more rapidly in the presence of residual liquid from a fungicide spray tank (Papadopoulos, 2006). There has to be a balance between the time frame within which the chemical is effective against its target pathogen and the its ultimate degradation to non-toxic components. Prochloraz is therefore applied as a split treatment, at two half-dose rates, the first one after casing, and the second one after first yield flush approximately 20 days later (Grogan, 2008).

Biological disease control of cultivated mushrooms

Many compounds of natural origin, such as antagonists, plant extracts, essential oils and their components, have been tested as control agents against edible mushroom diseases, demonstrating strong antimicrobial effects (Potočnik *et al*., 2005; 2010b; Soković *et al*., 2009; Tanović *et al*., 2006; 2009). Lactonase-producing bacteria, such as *Bacillus* species, have been examined for green mould prevention (Savoie *et al*., 2001). Based on these observations, biofungicide based on *Bacillus subtilis* has been approved against *T. agressivum* in French mushroom farms. In experimental growing rooms in Serbia, biofungicides based on *B. subtilis* and tea tree oil or their respective mixtures with prochloraz have shown considerable efficacy against *Cladobotryum* sp., although lower than prochloraz in single applications (Potočnik *et al*., 2010a). Biofungicide based on *B. subtilis* has demonstrated greater effectiveness in preventing green mould symptoms caused by *T. harzianum* than tea tree oil. *B. subtilis* combined with the fungicide exhibited less antagonism in its effectiveness against the pathogen than tea tree oil, and reduced the yield more than tea tree oil (Kosanović *et al*., 2013). Also, *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* proved to be very effective against *T. pleurotum,* pathogen of oyster mushroom (Nagy *et al*., 2012). Essential oils of oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) and their respective major components, carvacrol and thymol, have demonstrated very strong activity against T. *aggressivum* f. *europaeum*, *T. harzianum* and *T. atroviride*. Mint (*Mentha piperita*), which yields menthol as its major component, has also shown strong activity against various *Trichoderma* species (Soković *et al*., 2009). Oils of geranium (*Pelargonium graveolens*), cinnamon (*Cinnamomum verum*) and clove (*Eugenia caryophyllata*) also showed high activity against *Lecanicillium fungicola, M. perniciosa* and *Cladobtotryum* sp. (Tanović *et al*., 2006; 2009). An addition of tea tree (*Melaleuca alternifolia*) essential oil to oyster mushroom substrate or button mushroom casing results in strong inhibition of *T. harzianum* (Angelini *et al*., 2008; Kosanović *et al*., 2013).

Integrated disease management

Fungal pathogens are soil-borne fungi, and their primary source is black peat for casing soil. It has been noted that peat collected from lower layers seems to be their less likely habitat. Also, replacing the casing mixtures of clay, loam and humus with mixtures of sphagnum peat, sand and carbonate, has resulted in a considerable reduction in dry bubble incidence. The pH optimum for edible mushroom growth is alkaline whereas *Trichoderma* prefers acidic-neutral conditions (pH 5-7). This finding suggests that adjusting the pH of the substrate to 8-9 might slow down the growth of *Trichoderma*, resulting in a reduced spread of infection. In order to prevent a contamination from spreading, the application of calcium hydroxide onto the affected area on oyster mushorom supstrate has been suggested (Nagy *et al*., 2012). The environmentally friendly disinfectants based on active oxigen, and another one, based on colloidal silver and hydrogen proxide, have shown high antimicrobial activity (Todorović *et al*., 2012). Brown mushroom strain, cremini or portabella (*A. bitorquis*), is resistant to agents of bacterial blotch, dry bubble and green mould (Dragt *et al*., 1995). Also, wild strans of *A. bisporus* have been found to be significantly more resistant to pathogens than commercial ones (Olivier *et al*., 1997). Nevertheless, earlier fruiting strains are significantly less diseased by *Lecanicillium* (Largeteau *et al*., 2004). Antibiotics that are produced by button mushroom have a role in defence against the bacteria *L. fungicola* and *T. harzianum,* but not against *T. aggressivum* (Mumpuni *et al*., 1998). An important part of disease control can be achieved through improvements in farm hygiene, and by preventing the spread of spores by filtration and

by cooking out crops at the end of cycle. With regard to resistance development, harm to the environment, human and animal health, as well as an increase in production cost, attention should be focused on developing and implenting alternative methods of disease control.

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SEED TRANSMISSION OF *Xanthomonas vesicatoria* AND *Clavibacter michiganensis* subsp. *michiganensis* IN TOMATO AND *Xanthomonas euvesicatoria* IN PEPPER AND IMPLEMENTATION OF SEED DISINFECTION METHODS

Davide Giovanardi[1], Enrico Biondi[2], Maja Ignjatov[3], Katarina Gašić[4], Michele Ferrari[1], Set Perez[2], Radivoje Jevtić[3] and Emilio Stefani[1]

[1]Department of Life Sciences, University of Modena and Reggio Emilia, via Amendola 2, 42122 Reggio Emilia, Italy.
[2]DipSA, University of Bologna, via.le Fanin 44, 40127 Bologna, Italy.
[3]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia.
[4]Institute for Plant Protection and Environment, Teodora Drajzera 9, 11000 Belgrade, Serbia
E-mail: davide.giovanardi@unimore.it

ABSTRACT

Seed-borne bacterial pathogens of tomato and pepper are of major concern worldwide. *Xanthomonas vesicatoria* (Xv) and *Xanthomonas euvesicatoria* (Xe), the causal agents of bacterial leaf spot, and *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), the causal agent of tomato bacterial canker, are worldwide distributed, but the occurrence of the latter is usually erratic. In order to evaluate the risk of seed transmission and the relationship between seed contamination and disease outbreak, an extensive field trial has been put in place in 2013 for each pathosystem. Three artificially contamination levels were considered (1%, 5% and 15% or 20%, respectively in Italy and in Serbia), composed of 100 seedlings each. Disease outbreaks were monitored weekly during the growing season until harvesting and disease was quantified by means of AUDPC. Seeds were produced from each plot and analysed in order to assess their contamination level. Preliminary results of our studies showed that disease quantity caused by Xv, Cmm or Xe was directly correlated to the percentage of initial infection, according to AUDPC values obtained. Contamination rate of seed produced in diseased fields was not always correlated with disease quantity observed. A microbial consortium, a bacterial antagonist and plant polyphenols were assayed to assess their potential efficacy in seed disinfection: naturally contaminated tomato and pepper seeds were treated and sown. Pepper and tomato seedlings were inspected and analysed for the presence of bacterial spot. Preliminary results obtained show that none of the above mentioned treatments was able to eradicate the pathogen from seeds.

Key words: seed-borne bacteria, tomato, pepper, seed transmission, seed disinfection.

INTRODUCTION

Xanthomonas vesicatoria (Xv) and *X. euvesicatoria* (Xe) (Jones *et al.*, 2004) are the causal agents of bacterial spot of both tomato and pepper. Long-distance dissemination of those xanthomonads is ensured by means of contaminated seeds in trade (Carmo *et al.*, 2001). Bacterial spot is a widespread and economically very important disease of tomato and pepper. *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is the causal agent of tomato wilt and canker. The primary inoculum source for Cmm is contaminated seed (De

Leon *et al*., 2011; Chang *et al.* 1991) reported that one infected seed in 10.000 is able of initiating an epidemic. Cmm infections often result in high yield losses; in several cases, losses of between 50 and 100% have been reported. Xv, Xe and Cmm have been listed as A2 quarantine pests by EPPO.

This preliminary study was aimed to assess the impact of the diseases in the field, after an experimental inoculation with Xv, Cmm and Xe on tomato and bell pepper plants in different plots, and to evaluate the transmission rate from the infected plants to the seeds. In this study we also investigated the efficacy of different treatments, to reduce pathogen contamination in tomato and pepper seed, by treatments with a natural plant polyphenols, a microbial consortium and an antagonistic bacterium, specific for Xv.

MATERIAL AND METHODS

For each pathosystem, three fields of 96 plants each were set, and the plants of any field were randomly marked and inoculated in order to obtain 3 different percentages of initially inoculated plants: 1, 5 and 15% in Italy (industrial tomato), and 1, 5 and 20% in Serbia (table tomato and bell pepper). High susceptible cultivars to bacterial diseases were used: industrial tomato, cv. VF10; table tomato, cv. Jabučar and bell pepper, cv. Amphora. Experiments with Xv, Xe and Cmm were conducted in confined experimental field.

Experimental inoculation and phytopathometric evaluation

Tomato and pepper seedlings were transplanted in the fields, following the best agricultural practices in place for Italy and Serbia. For tomato inoculation, strains IPV-BO 2684 of Xv (Italy), KFB29 of Xv (Serbia), DLS 598 of Cmm (Italy) and for pepper inoculation the strain MI-A-6 of Xe, were routinely grown on GYCA (Dye, 1962) for 48 hours at 27°C. Five weeks after transplanting, each plant was experimentally inoculated by spraying a water suspension containing the pathogen (ca. 10^8 CFU/mL). Each inoculated plant was sealed in a polythene bag (PE) overnight, which was removed the early next morning. The first phytopathometric readings were done at symptoms appearing and were carried out weekly. The disease severity of tomato and pepper plant affected by xanthomonads was evaluated using a descriptive scale ranging from 0 to 4: 0= no symptom; 1= 1-10 spots on 1-3 leaves; 2= 11-30 spots on 4-10 leaves; 3= more than 30 spots and some confluent necrosis on 5-20 leaves; 4= confluent necrosis on more than 20 leaves or branch desiccation. In case of Cmm infections, the disease severity on leaves (percentage of symptomatic leaves) was evaluated on each tomato plant on the basis of 5 disease severity classes (0, 5, 10, 25 and 50%). Disease score was calculated as $\sum$ of Q = Severity x Incidence. Area under the disease progress curve (AUDPC; Van der Plank, 1963) was then calculated according to Madden *et al.* (2007). Data were collected and statistically evaluated from the first observed symptoms to the last assessment before harvesting. In case of pepper and table tomato, readings were done during a longer time span, since harvesting was done gradually.

Crop harvest and seed extraction

Tomato and pepper seeds were produced according to common commercial procedures. For both tomato cultivars, the seed was extracted following the fermentation technique.

Seed analyses for the estimation of seed infection rate

For each pathosystem, ten samples of 100 seeds each, belonging to each infected field, were soaked in 3 mL of sterile PBS-Tween 20 (0,05%) for 14 hours at 4°C (see ISTA rules). The samples were then crushed for 2 minutes, the extraction liquid was centrifuged at 10.000 *g* for 20 min at 4°C and the pellet was resuspended in 2 mL of sterile PBS-Tween 20. DNA was extracted from seed macerates using the DNeasy Plant Mini kit (Qiagen) and assayed using the protocol of Koenraadt *et al.* (2009): Bs-XeF and Bs-XeR primer pair for Xe and Bs-XvF and Bs-XvR primer pair were used to detect Xv. DNA isolated from Cmm infected seed was assayed according to Dreier *et al.* (1995). The analyses were repeated 5 times in different days (5 replicates), for a total of 5000 seeds, in order to statistically assess the seed contamination rate.

Biological treatments of seed

Tomato seed, cv. Jabučar and pepper seed, cv. Amphora, naturally contaminated by Xv and Xe, respectively, were used. The following compounds were tested: a commercial microbial consortium and a commercial plant polyphenols on both tomato and pepper seeds, as well as a bacterial antagonist on tomato

seed. The microbial consortium (Micosat F, CCS Aosta, Italy) contained: *Glomus* spp., *Trichoderma* spp., *Agrobacterium radiobacter*, *Bacillus subtilis*, *Streptomyces* spp. Treatment was done according the manufacturer's indications: seed was dipped in a water suspension of the consortium, calculating 4.5 g/kg of seed. A commercial plant polyphenols based on tannins (AGRITAN, Silvateam, San Michele di Mondovì, Italy) was used. Treatment has been done according the manufacturer's indications, by dipping seeds in a 10 g/L polyphenol solution in deionized water. A strain of *Pseudomonas synxantha* (DLS A65) active *in vitro* against Xv was preliminary assayed to control Xv on tomato seeds. Treatment has been done by dipping seed in a bacterial suspension of 10^8 CFU/ml. For treatments, seed was kept soaking for 90 min in a rotary shaker at 90 rpm, dried in an incubator at 30°C (with fan) overnight in the dark and stored in a seed storage room 1 month before sowing. Untreated seeds were used as a positive control.

Seed germination and disease assessment

In order to assess the effects of biological treatments on seed quality and its efficacy in seed sanitation, three replicates, consisting of 100 seeds for each treatment, were assayed in each of the three following tests. Germination *in vitro* was done according to ISTA rules. Seeds were placed on top of two layers Whatman n° 5 filter paper, moistured with 5 ml of sterile distilled water in Petri dishes. Petri dishes were placed at 25°C in the dark. Germination counts were assessed every day, up to 14 days. Germination test on blotter was carried out in a growing chamber, at 28-30°C and RH up to 75%. In pot tests, seeds were sown into pots containing a steam sterilized peat for seedling production. Growing chamber conditions were kept as above. Disease symptoms were daily monitored up to 28 days. In case of no symptoms development within 4 weeks, a stem segment (~2cm) of each seedling within the same replicate was collected and placed in a Stomacher Bag with 30 ml of sterile NaPBS buffer (137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 1,8 mM KH_2PO_4, pH = 7.2). All samples were crushed by hammering and stored at room temperature for 30 minutes. The washing fluids were then centrifuged and DNA was extracted by using DNeasy Plant Mini kit (Qiagen). The DNA was extracted from seed macerates using DNeasy Plant Mini kit (Qiagen) and assayed using the protocol of Koenraadt *et al.,* (2009). Primers used were: Bs-XeF and Bs-XeR for Xe and Bs-XvF and Bs-XvR for Xv.

Statistical analysis

All measurements were performed in triplicates. Analysis of variance (ANOVA, Tukey's test, $P \leq 0.05$) was applied using GraphPad Prism 6.0 software (La Jolla, California, USA).

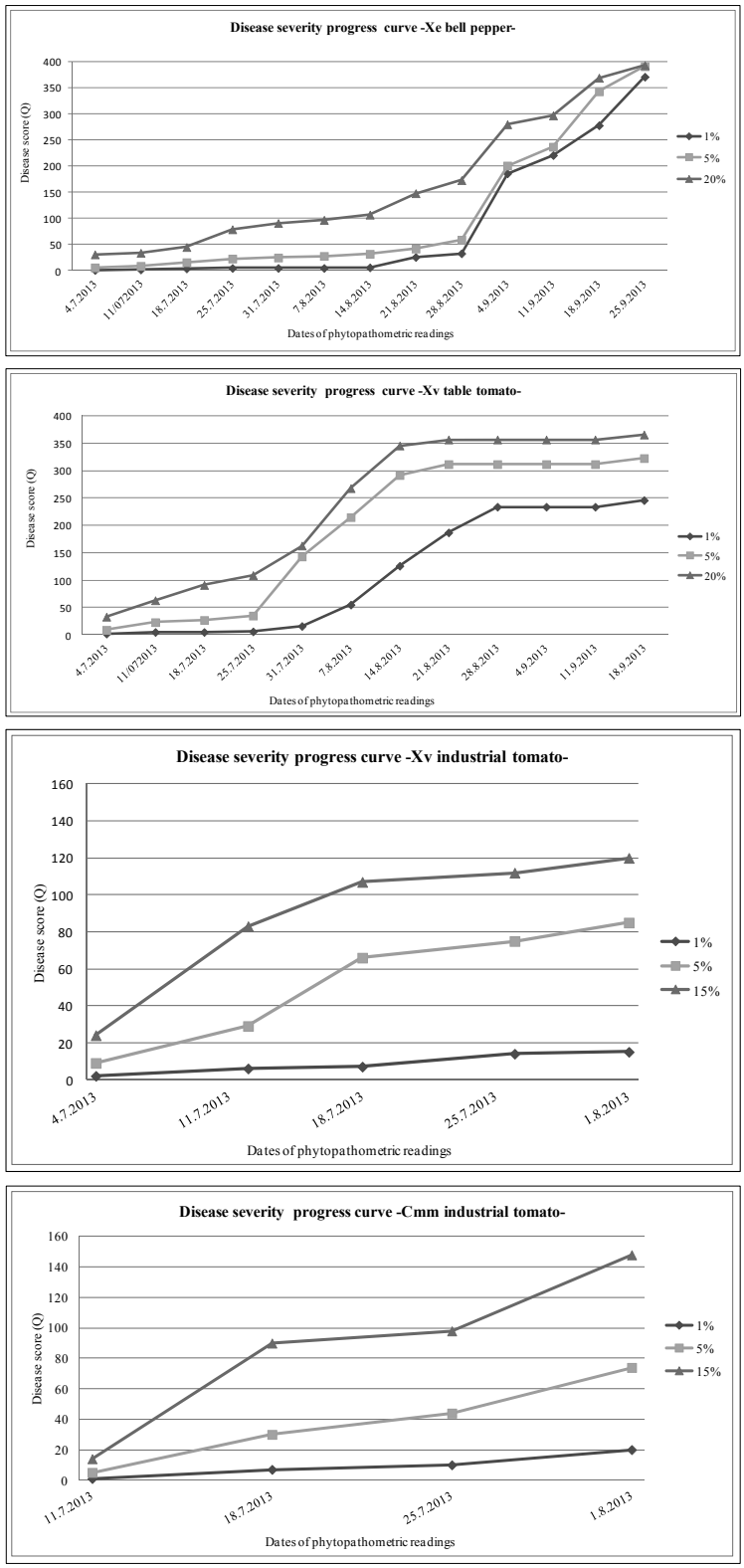
RESULTS

Phytopathometric evaluation of field experiments

For the experiments that were performed in Italy, the increase of the disease progression curve calculated for Xv and Cmm in industrial tomato was directly correlated to the percentage of initial infections; disease symptoms appeared 2 and 3 weeks after inoculation, respectively, and increased until the last survey (Graph 1). For industrial tomato plants inoculated with Xv, the AUDPC of the field with 1% initial infection was approximately six and ten times lower than that of the fields with 5% and 15% respectively. As regards the AUDPC obtained for Cmm from the field with 1% of initial infection, it was approximately four and ten times lower than that of the fields at 5% and 15%, respectively (Table 1). In Serbia, bacterial spot symptoms on table tomato and bell pepper appeared 2 weeks after the experimental inoculation and increased until the last survey (Graph 1). AUDPC value for Xv in the field at 1% of initial infections was approximately two times lower than that of fields at 5% and 20%. For Xe, the AUDPC referred to 1% of initial infection was approximately two and three times lower than that of the fields at 5% and 20%, respectively (Table 1).

Table 1. AUDPC values obtained in the different pathosystems considered (according to Madden *et al.* 2007).

Pathosystem	AUDPC values		
	Initial contamination rate (experimental)		
	1%	5%	20%
Xv-table tomato	8589	15074	18788
Xe-bell pepper	5743	8522	13632
	Initial contamination rate (experimental)		
	1%	5%	15%
Xv-industrial tomato	249	1512	2654
Cmm-industrial tomato	196	812	1932



Graphic 1. Disease severity progression curves over the time of each pathosystem considered in this study. In the legend, percentage value indicates initial percentage of inoculated plants per field.

Molecular analyses of seeds

The molecular analysis of seeds, by means of PCR protocol, did not result in the detection of Xv, in both industrial and table tomato (in Italy and in Serbia). On the contrary, seeds produced in Cmm contaminated plots were found to be all positive by PCR. Seed samples obtained from field plots inoculated with Xe at 1, 5 and 15% level resulted in PCR positive by 78, 96 and 96%, respectively.

Biological treatments of seed

Germination tests on blotter, performed with tomato and pepper seeds after biological treatments, did not show significant differences to the untreated ones. In *in vitro* experiment, the germination rate of tomato seeds treated with the microbial consortium and commercial plant polyphenols showed an apparent, but not significant increase compared to that of the untreated. The treatment with the bacterial antagonist DLS A65 significantly affected the germination rate of tomato seeds (Table 2). Germination tests *in vitro*, performed with pepper seeds after a treatment with the microbial consortium, was not different to untreated control, on the contrary, a treatment with commercial plant polyphenols decreased the germination by approximately 10%, if compared to untreated seeds. Such decrease was significant ($P \leq 0.05$). No symptom development was ever observed in both tomato and pepper seedlings until 28 days. Interestingly, PCR tests performed on same seedlings, confirmed the presence of Xv and Xe in tomato and pepper seeds, respectively.

DISCUSSION

In this study, we demonstrated during our field experiments a positive correlation between percentages of initial infection and disease progression and quantity caused by Xv, Xe and Cmm, as shown by the AUDPC value obtained. In addition, we highlighted differences in the AUDPC values obtained in industrial tomato fields and in table tomato plots: those differences might be explained by the length of cultivation, remarkably longer for table tomato (7-8 weeks longer) than for industrial tomatoes. The same for bell pepper, since monitoring and harvesting of peppers continued for additional 8 weeks, after industrial tomato harvesting day. Among the different pathosystems, contamination rates of tomato seed produced in affected plots were not correlated with disease quantity observed and measured in the fields. In particular, no contamination rate of Xv was found in both table and industrial tomato seeds, although the disease observed was remarkably severe and present on all aerial parts: leaves, fruits, petioles and stems. In contrast, pepper and tomato seeds, respectively produced in Xe and Cmm contaminated plots, were all found PCR positive. Further work is necessary to deeply investigate the pathogen transmission from plant to seed and from seed to plant by means the setup of extensive field trials using seed produced during this study. Further experiments are underway to assess the effect on the bacterial cells viability (Xv and Cmm) of the fermentation process during tomato seed extraction, which supposedly reduced the bacterial load.

Biological seed treatments with plant/fungal extracts apparently enhanced the germination rate *in vitro* and on blotter for tomato seed. On the contrary, no effect on the germinability was observed for pepper seeds. No bacterial spots occurred during the pot test on tomato and pepper seedlings; however, asymptomatic plantlets, collected and analyzed with PCR assays, showed that bacterial inoculum was present. Therefore, bacteria from seeds moved acropetally and colonised the seedlings: there they may survive as residents or increase the populations in seedlings until they reach the leaves without causing symptoms (Silva *et al*., 2013). Results of the biological seed treatments showed that they were not effective

Table 2. Germination rate *in vitro* and on blotter of tomato seed cv. Jabučar and pepper seed cv. Amphora after treatments. Different letters within columns denote significant differences according to the Tukey's test ($P \leq 0.05$).

	Treatment	Germination (%)	
		in vitro	blotter
Tomato cv. Jabučar	Microbial consortium (Micosat F, CCS Aosta, Italy)	98.67^{A}	96.67^{A}
	Plant polyphenols (AGRITAN, Silvateam, Italy)	92.67^{A}	91.67^{A}
	Bacterial antagonist	78.33^{B}	$80,67^{A}$
	Untreated	86.67^{AB}	85.67^{A}
Pepper cv. Amphora	Microbial consortium (Micosat F, CCS Aosta, Italy)	97.00^{A}	92.00^{A}
	Plant polyphenols (AGRITAN, Silvateam, Italy)	84.00^{B}	85.67^{A}
	Untreated	96.33^{A}	85.67^{A}

in eradicating the pathogenic bacteria associated with seeds. Further studies are needed to check, if such plant polyphenols or beneficial microbes might have a role in inducing of resistance. They might also enhance the germinability and the performance of seeds. Additionally, they could be taken into consideration to increase plant productivity of tomato and pepper crops. Nevertheless, new approaches in sanitation methods are needed to ensure efficient seed sanitation/disinfection, together with an optimization of formulations and application procedures related to such innovative bioproducts.

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SEED TRANSMISSION OF *Acidovorax citrulli:* IMPLEMENTATION OF DETECTION IN WATERMELON SEEDS AND DEVELOPMENT OF DISINFECTION METHODS

Davide Giovanardi, Michele Ferrari and Emilio Stefani

Department of Life Sciences, University of Modena and Reggio Emilia, via Amendola 2, 42122 Reggio Emilia, Italy.
E-mail: davide.giovanardi@unimore.it

ABSTRACT

Acidovorax citrulli is a seed-borne pathogen and the causal agent of bacterial fruit blotch of cucurbits. It is listed as an A1 quarantine pathogen by EPPO. Seed certification is based on the availability of a sensitive and specific pathogen detection in seed lots: this is a must for an effective disease management strategy. Therefore, an effective DNA extraction and purification procedure is a critical issue to ensure a robust PCR analysis. Pathogen detection in seed lots has been implemented by testing different known contamination levels by *Acidovorax citrulli***.** Initially, two different sample preparation methods have been tested: a) Overnight soaking; b) Hammering of dry seeds, followed by three different manual DNA extraction. Each DNA sub-sample obtained has been analysed with two different primers sets, SEQID3/SEQID4 and WFB1/WFB2, to evaluate the capability to detect the pathogen. Results showed that a DNA extraction and purification procedure, based on soaking the seeds, followed by the use of the DNeasy Plant Mini kit (Qiagen) on the washing fluids gave the highest amount of DNA, sufficient to increase the detection threshold of the pathogen. This will allow the improvement of current detection procedures.

Furthermore, naturally contaminated watermelon seeds were treated through different methods, in order to achieve a possible sanitation or eradication of *Acidovorax citrulli*: a bacterial antagonist, a microbial consortium, a plant polyphenol. Our results showed that treated seeds were only partially disinfected, and the pathogen was not eradicated after any of the methods used.

Key words: *Acidovorax citrulli*, watermelon, pathogen detection, seed disinfection.

INTRODUCTION

Bacterial fruit blotch of cucurbits (BFB) is a relatively new disease and became a severe problem in watermelon in the late 1980s. It was first reported to occur in Australia in 1988 (Wall & Santos, 1988) and observed in U.S. commercial watermelon fields in 1989 (Latin & Rane, 1990). Since then, BFB has locally spread worldwide. BFB can be devastating for growers, with fruit losses reaching 80-100%, in watermelon (Latin & Hopkins, 1995; Schaad *et al.,* 2003), and in recent years, in melon (Burdman *et al.,* 2005). The causal organism is the Gram negative, non-fluorescent, rod-shaped bacterium, *Acidovorax citrulli* (Acit) (Schaad *et al.,* 2008). Acit is a seed-borne pathogen and infects seeds, which represents the most important source of primary inoculum for BFB epidemics (Latin & Hopkins, 1995; Walcott & Gitaitis, 2000).

Strategies, which exclude Acit from seeds, are the main issue to avoid further phytosanitary problems to the crop during the growing season. The need for an efficient, fast and reliable detection method is widely

required and several methods, mainly PCR-based, have been proposed (Walcott & Gitaitis, 2000; Schaad *et al.,* 2000; Song *et al.,* 2003). Melon seed was included in this study, in order to compare detection threshold obtained from watermelon seed to another cucurbit species, being BFB a serious threat for watermelon and, in the recent years, for melon (Burdman *et al.,* 2005).

The above-mentioned PCR-based protocols present some limitations: in particular, seed represents a far more difficult matrix to analyse, due to presence of several contaminants, coating chemicals, PCR inhibitors as (but not only) a high starch content in the cotyledons. Therefore, DNA extraction and purification are critical to ensure a reliable PCR analysis of seed lots for certification or other purposes. This study was aimed to implement a suitable and accurate seed sample preparation strategy, followed by a comparison of 3 different DNA extractions Kits to be used prior to a PCR assay.

Seed treatments with biomolecules or microorganisms have been reported to reduce disease severity and increase seed germination (Gupta *et al.,* 2002; Jensen *et al.,* 2004). Microbial consortia, plant polyphenols and an effective bacterial antagonist were assayed for their possible effect to reduce the seed-borne inoculum. This study aimed to implementing an effective BFB management, based on a highly sensitive molecular detection of the pathogen and developing a biological seed treatment, which might significantly reduce the seed-borne inoculum.

MATERIAL AND METHODS

Sample preparation for analysis: calibrated contamination with Acit

Experimentally infected watermelon seeds, cv. Charleston Gray and melon seeds, cv. Silver Star, were produced. Five hundred seeds for each cucurbit were dipped into an Acit bacterial suspension prepared at the concentration of $1x10^8$ CFU/ml, spectrophotometrically adjusted, and followed by vacuum infiltration (-60 cm/Hg) for 90 minutes. Seeds were then dried in an incubator at 25°C (with fan) overnight in the dark**.** Number of CFU per seed was determined by taking 10 seeds per crops and incubating them for 2 hours in 1 ml of PBS, added with 0.2% Tween 20 (PBST). Each seed was ground in a sterile mortar with a pestle, and 100 µl of the seed macerate were used to prepare 10-fold dilution series, up to 10^4 dilution and plated onto nutrient sucrose agar (Crosse, 1959), supplemented with 250 ppm of cyclohexymide and 200 ppm of ampicillin (NSA-250). Each dilution was represented by 6 drops of 10 µl. Agar plates were then incubated at 28°C for 4-8 days, followed by counting the colonies grown, so to precisely calculate the number of CFU per seed. Experimentally contaminated seeds were used to obtain different contaminated batches of seeds: 1 contaminated seed in 10; 1 in 100; 1 in 1000. Negative control (500 seeds proved to be Acit negative) and positive control (500 seeds into PBST spiked with Acit, to obtain a final concentration of 10^6 CFU/ml) were also assayed. Both overnight soaking and direct hammering of dry seeds sample preparation methods were tested.

Sample preparation for analysis: soaking

Each contaminated batch was placed overnight in a PBST soaking buffer (2 ml of soaking buffer per gram of seed) and shaken at 90 rpm at room temperature on a rotary shaker. The seed washing fluids were centrifuged for 5 minutes at 650 x *g* to collect seed debris, followed by a centrifugation at high speed of the resulting supernatant for 20 minutes at 12.000 x *g*, to obtain a final pellet with the target bacteria. Pellets obtained were finally resuspended in 1 ml of sterile water prior to DNA extraction and purification.

Sample preparation for analysis: hammering

Dry seeds of each contaminated batch were crushed by hammering and placed into PBST soaking buffer (3 ml of soaking buffer per g of seed). The crushed seed samples were then shaken for 3 hours at 90 rpm at room temperature on a rotary shaker; the extraction fluids were initially centrifuged at 650 x *g* for 5 minutes to collect seed debris. The supernatants were then centrifuged at 12.000 x *g* for 20 minutes, and each of resulting pellets was resuspended in 1 ml of sterile water, prior to DNA extraction and purification.

DNA extraction

Three different extraction procedures were tested: DNeasy Plant Mini Kit (Qiagen), DNeasy Blood and Tissue (Qiagen) and Wizard Magnetic 96 DNA Plant

System (Promega). DNA extraction procedures were done according to the manufacturer's instructions.

PCR assay

DNA, extracted and purified from each sample, was amplified in parallel with two different primers sets: SEQID3/SEQID4 (Schaad *et al*., 2000) and WFB1/WFB2 (Walcott & Gitaitis, 2000) The primers of Schaad *et al*. (2000) were used in a protocol modified as follows: amplifications were carried out in a final volume of 25 µl, containing 1x PCR Buffer (Promega), 1.5 mM of $MgCl_2$ (Promega), 0.2 µM of each primers, 1.0 U Go Taq® G2 Flexi DNA polymerase (Promega), 200 µM each dNTP (Promega) and approximately 50 ng of target DNA. PCR reactions were performed using the following conditions: denaturation at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds and elongation at 72°C for 1 minutes, with a final elongation step of 7 minutes at 72°C. DNA amplicons obtained from both amplifications were run in a 2% agarose gel, stained with ethidium bromide and observed with the BioDoc Analyze (Biometra, Göttingen, Germany).

Biological seed treatments

Naturally contaminated watermelon seeds were subject to 3 different biological treatments, in order to test their efficacy in seed sanitation from Acit. Untreated seeds were used as a positive control.

The following compounds were tested: a commercial microbial consortium, plant polyphenols and a bacterial antagonist. The microbial consortium (Micosat F, CCS Aosta, Italy) was composed by: *Glomus* spp., *Trichoderma* spp., *Agrobacterium radiobacter*, *Bacillus subtilis*, *Streptomyces* spp. Treatment was done according to the manufacturer's indications: seed was dipped in a suspension of the microbial consortium, calculating 4.5 g/kg of seed. Commercial plant polyphenols based on tannins (AGRITAN Silvateam, San Michele di Mondovì, Italy) were used according to the manufacturer's suggestions at a concentration of 10 g/l in deionised water. A bacterial antagonist (*Pseudomonas synxantha*, strain DLS 65, from UNIMORE culture collection) was used to prepare a suspension of 10^8 CFU/ml. Treatment was done by dipping seeds in the bacterial suspension, keeping the seeds soaking for 90 minutes on a rotary shaker at 90 rpm. Seeds were then dried in an incubator at 25°C (with fan) overnight in the dark and stored in a seed storage room 1 month before sowing.

Germination and disease incidence assay

Germination tests *in vitro* were done according to ISTA rules. Three replicates of 100 seeds were used for germination test. One hundred seeds were placed on top of two layers Whatman n° 5 filter paper, moistured with 5 ml of sterile distilled water in Petri dishes. Petri dishes were placed at 25°C in the dark. Germination counts were assessed every day, up to 14 days. Germination test on blotter was carried out in 3 replicates of 100 seeds for each treatment. Germination counts were assessed every day, up to 14 days. Greenhouse temperature was kept at 28 to 30°C and the relative humidity at 75%. Pot test was assayed on triplicates of 100 seeds for each treatment; seeds were sown into pots containing a steam sterilized peat for seedling production. Greenhouse condition was kept as above (germination test on blotters). Disease symptoms were daily evaluated up to 28 days. Symptomatic seedlings were collected, placed in a Stomacher Bag and homogenised in PBST buffer. The washing fluids were filtered with a sterile gauze, transferred in a centrifuge vial and centrifuged for 5 minutes at 1.250 x *g* to pellet soil and plant debris. The supernatants were then centrifuged at 12.000 x *g* for 20 minutes; the pellets were resuspended in 1 ml of sterile water. DNA was then extracted and purified using the DNeasy Plant Mini kit (Qiagen). The DNA isolated and purified was assayed, according to the modified protocol of Schaad *et al*. (2000) described above, in order to confirm the presence of Acit.

Statistical analysis

All tests were performed in triplicates. Data were presented as mean ± SD for each treatment. Univariate analysis of variance (ANOVA) with Tukey post-test was applied using GraphPad Prism 6.0 software (La Jolla, California, USA) when multiple comparisons were performed. The differences were considered significant when $p \leq 0.05$.

RESULTS

Detection assay of Acit

The level of contamination calculated for melon and watermelon seeds was consistently assessed about 10^3 CFU/seed. PCR detection threshold obtained using the DNeasy Plant Mini Kit (Qiagen) allowed detecting 1 artificially contaminated seed in 1000 for each species.

This was achieved with both soaking and hammering of seeds and using both primers pairs. Other procedures were not such sensitive (Table 1). Other combinations of seed treatment, DNA extraction and PCR detection were, in general, either less sensitive or less specific.

Biological seed treaments

Germination test *in vitro* showed a slight increase of germination rate by using the microbial consortium and the commercial plant polyphenols: 2 and 4% respectively, compared to untreated seeds. On the contrary, treatments using the bacterial antagonist significantly ($p \leq 0.05$) reduced the germination rate by 9%. On blotter, effects of treatments on germination were not significant ($p \geq 0.05$), and showed an increase of germination rate by using the microbial consortium and the commercial plant polyphenols (4 and 6% respectively), compared to untreated seeds. Finally, treatment with the bacterial antagonist had a similar value as untreated seeds. Regarding disease development on seedlings, assessed after all treatments, our data showed a significant reduction (27%, $p \leq 0.05$) of symptomatic seedlings, when the bacterial antagonist was applied to seed. The application of the microbial consortium and the commercial plant polyphenols apparently reduced the percentage of diseased seedlings by 5 and 12% respectively, compared to the untreated control (Table 2).

DISCUSSION

Hammering of dry seeds might be a suitable method to allow bacteria present inside seeds to escape, since the thick seed coat is a strong barrier. This method proved to be very time consuming (~ 1 hour per 1000 seeds) and not feasible in routine seed analysis, although the matrix obtained by soaking was compatible with different DNA extraction methods. Overnight soaking is the easiest and fastest handling method. Detection threshold of Acit, as obtained by simplex-PCR, confirmed that DNA extraction with DNeasy Plant Mini Kit (Qiagen) gives the highest value (1 artificially contaminated seeds in 1000) in watermelon and melon seeds, assayed by means of soaking and hammering sample preparation. In order to further increase the detection sensitivity and the feasibility to analyse a sample size of 10.000 seeds (ISTA recommendation), an implementation and validation of a multiplex Real-Time TaqMan PCR assay is now in progress and is based on the results obtained from our studies on sample preparation and DNA extraction.

Biological seed treatments with plant/fungal extracts showed to reduce the percentage of symptomatic seedlings and slightly increasing the germination percentage. Bacterial antagonist DLS 65 used in this study significantly decreased the percentage of symptomatic seedlings by 27%, without affecting the germinability on blotter and in soil (data not shown). Seed treatments tested were not able to eradicate the bacteria, and this

Table 1. Detection threshold (1 contaminated seed in x seeds) by simplex PCR with SEQID3/SEQID4 (Schaad *et al.,* 2000) and WFB1/WFB2 (Walcott *et al.,* 2000) primers. DNA extraction methods: PMK= DNeasy Plant Mini kit (Qiagen); B&T= DNeasy Blood and Tissue (Qiagen); Wizard 96 = Wizard Magnetic 96 DNA Plant System (Promega).

		Melon		Watermelon	
		SEQID3/4	WFB 1/2	SEQID3/4	WFB 1/2
PMK	Soaking	1:1000	1:1000	1:1000	1:1000
	Hammering	1:1000	1:1000	1:1000	1:1000
B&T	Soaking	1:1000	1:1000	Negative	1:100
	Hammering	1:100	1:1000	Negative	1:1000
Wizard 96	Soaking	1:1000	1:100	Negative	1:1000
	Hammering	1:1000	1:100	Negative	1:100

Table 2. Germination percentage *in vitro* and on blotter and percentage of diseased watermelon seedlings grown after treatments. Data presented as mean ± SD for 3 replicates for each treatment. An asterisk indicates that data are significant ($p \leq 0.05$).

Treatment	Germination %		Diseased seedlings %
	in vitro	blotter	
Microbial consortium (Micosat F, CCS Aosta, Italy)	92.33 ± 2.08	90.00 ± 1.00	56.00 ± 3.00
Plant polyphenols (AGRITAN, Silvateam, Italy)	94.00 ± 3.00	91.67 ± 2.52	59.67 ± 2.65
Bacterial antagonist strain DLS 65	*81.00 ± 1.53	84.67 ± 2.00	*46.67 ± 2.52
Untreated	90.33 ± 4.35	86.00 ± 6.02	63.33 ± 4.16

could be explained by the localization of Acit in the embryos of watermelon seeds (Dutta *et al*., 2012); nevertheless such treatments can be inducers of resistance and can also enhance and boost the performance of seeds either in processing and planting equipment or mitigate environmental stress. Additional research is planned to improve the seed treatment protocol, with the application of a new formulation obtained by addition of methylcellulose to the bacterial antagonist suspension prior to treatment, in order to achieve a better adhesion of the seed coating.

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THE RESISTANCE OF DIFFERENT POTATO CULTIVARS ON YELLOW CYST NEMATODE (*Globodera rostochiensis* pathotype Ro1)

Dobrivoj Poštić[1], Đorđe Krnjaić[1], Zoran Broćić[2], Nebojša Momirović[2], Rade Stanisavljević[1], Lana Đukanović[1] and Ratibor Štrbanović[1]

[1] Institute for Plant Protection and Environment, 11000 Belgrade, Serbia
[2] Faculty of Agriculture, 11080 Belgrade, Serbia
e-mail: pdobrivoj@yahoo.com

ABSTRACT

Resistance testing of different potato varieties to the Golden Potato Cyst Nematode (GPCN) *Globodera rostochiensis*, pathotype Ro1 was carried outin 2014 in the locality of Krupanj on Jagodnja Mountain in Western Serbia. The research used two varieties of potatoes susceptible to GPCN–Ro1 (Desiree and Lusa), that have exhibited a high degree of sensitivity to GPCN - Ro1. Varieties declared as resistant to GPCN-Ro1 (Karlena, Pirol, Lady Claire, Crips4all and Arizona) have exhibited a high degree of resistance to this specific pathotype. The results indicate practical importance of growing resistant varieties in the infected area in the Mačva District with the aim of suppression and eradication of quarantine nematode *Globodera rostochiensis* pathotype Ro1.

Key words: *Globodera rostochiensis,* nematode, potato, cyst

INTRODUCTION

Potato Cyst Nematodes (PCN) are presentin the Republic of Serbia, since the year 2000 (Golden PCN *Globodera rostochiensis,* Wollen, 1923, Behrens, 1975) and 2005 (White PCN *Globodera pallida*, Stone 1973) respectively, and their presence have been verified in several locations. PCN are quarantine nematode species in many countries around the world (Lehman, 2002). Yield losses in Europe caused by PCN are estimated at about 9% of overall potato productionin the region (Turner and Rowe, 2006). In total Serbian food production potatoes of great significance. The great economic importance of potato derives from the area planted - about 78,000 ha, withan average yield for the period 2003-2013 reaching the levelof 11,3 t/ha (Statistical Yearbook of Serbia, 2013). Recorded average yields in Serbia are significantly behind the average European or World yields ranging from 37,0 to 50,0 t ha^{-1} (FAO, 2013). The commercial potato production in Serbia is established on 50.000-60.000 ha with an average yields between 15-25 t ha^{-1}, level that is still far from the standards in modern agricultural production.

Golden species *Globodera rostochiensis* (GPCN) was foundin 2000 in the potato crops in Jagodnja, Tara (Ponikve), Javor and Sjenica (Krnjaić et al., 2002, 2005ab, 2008). GPCN was later recorded in other localities in Moravički County and Mačva District. In the region of Javor Mountain in the year 2005 the presence of white species *Globodera pallida* (WPCN) was detected in Kušići, localities Šanac and Kladnica, and in two localities (Ograđenik and Milatovići) mixed populations of GPCN and WPCN (Krnjaić et al., 2005b). In order for the strategy of suppression and eradication of PCN to be efficient early detection of either species or mixed population, determination of pathotype of each of these species, introduction of potato varieties resistant to pathotype into the crop rotation system are essential, as well as permanent implementation of antinematodal measures including the use of systemic nematicides, such as Aldicarb (Temik), Entoprophos (Mocap), Fosthiozat (Nematos), Oxamyl (Vydat), while the liquid fumigants

remain prohibited due to toxicity of their residuesin soil and water as of year 1990.

Crop rotationis very important agro-technical measurein suppression anderadication of PCN. The most commonly recommended pause for growing potatoes in areas previously infected with PCN is 3-7 years. According to recent researches vitality of invasion content of GPCN in the soil, in the absence of the host plant, can be held for up to 20 years (Pridannikov et al., 2006), which questions or relativize the application of simple crop rotation, without introductionof PCN resistant potato crops in the crop rotation system and application of nematicides, together (Trudgill et al., 2003). In relation to environmental factors resistance of invasion contentin the cysts (eggs, J2) is extremely high. Pridannikovet et al. (2006) found that homogenate from eggs of GPCN retains viability even after twenty minutes of immersion in boiling water (at 100 °C) and even after five cycles of cooling (freezing) at -20°C and then heating up to 22 °C.

Krnjaić and Poštić (2009) reported that crop rotation including grass-legume mixtures, no matter how long it lasted, does not provide extinguishing GPCN infestation, because the potato tuber reproduced in these conditions will allow GPCN to survive and maintain activity. The same authors recommend crop rotation with row crops (except plant species from the fam. Solanaceae), with plowing and othe ragro-technical measures extracting residual tubers or at least moving them closer to the surface in shallow soil layer (5-10 cm depth), allowing winter temperatures for the period of 5-6 years to reduce PCN activity leading in the end to their extinction and in this way breaking the reproductive chain of PCN. There are great choices of GPCN resistant and tolerant varieties of potatoes, especially regarding pathotype Ro1 (Poštić et al., 2013a). On the other hand selection of WPCN resistant potato varieties, resistant on one or more types (3), is very limited and unreliable due to the high aggressiveness of this type of PCN and fast reduction in resistanceof selected potato varieties to this type of PCN.

Martin et al., (2004) reported that in England has not yet been selected no commercial potato varieties resistant to pathotypes of WPCN. In the US the limited number (16) of GPCN- Ro1 resistant potato varieties is used in the production and the system works very effectively (Trudgill et al., 2003). Surfaces infected with WPCN or mixed populations of WPCN and GPCN must be subjected to many years of crop rotation to final closure of infection. In our conditions in the areasinfectedwith GPCN - Ro1 also is recommended to use varieties of potato resistant to GPCN - Ro1 (Krnjaić and Poštić, 2009; Poštić et al., 2012, 2013ab).

The aim of this study was to point out to importance ofcultivation of GPCN *Globodera rostochiensis* pathotype Ro1 resistant potato varieties, for the suppression and eradication of these quarantine nematodes.

MATERIAL AND METHODS

In order to determine the resistance of the tested varieties in the presence of the population of the Golden Species *G. rostochiensis* pathotype Ro1 (GPCN), experiment is set on a plot with an infected locality Planina, municipality Ljubovija on the Jagodnja Mountain in the Mačva Districtin Western Serbia (759 m a.s.l., 44°19 '33 „N, 19°20'33" E). To perform a field experiment two (2) GPCN - Ro1 susceptible varieties of potato were used - Desiree and Lusa (as a control varieties) and five (5) GPCN - Ro1 resistant potato varieties (Karlena, Pirol, Lady Claire, Crips4all and Arizona).

Soil on the experimental field belongs to the acid and brown podzols. According to the humus contentin the surface layer of 3.40% (Table 1), the plot soil is very well provided. Total nitrogenis 0.27% defining it as rich soil. This soil also shows highly acidic reaction, in the H2O as solvent pH value is 4.35, and in KCL 3.80. The top layer of the soil is well provided with readily available phosphorus (19.96 mg/100g of soil). The content of easily accessible K2O is 36.04 mg/100g of soil, thus classifying this soil as very well provided. The content of soluble potassium is insufficient to achieve high yields of potatoes and must be compensated through adequate fertilization. According to the carbonate content soil belongs to weakly calcareous soils.

Size of the elementary plot was 10.5 m^2, while the trial field area was 63 m^2. Planting tubers was carried out on 03.05.2014, according to the plan of planting - 40 tubers

Table 1. Properties of soil at the experimental plot (Krupanj)

Depth (cm)	Tipe of Soil	CaCO3 %	pH		Humus %	N %	mg/100g Soil	
			H2O	nKCl			P2O5	K2O
0-30	Brownpodzol	0,69	4,35	3,80	3,40	0,27	19,96	36,04

of each variety (4 rows of 10 tubers), row spacing of 0.7 m and the distance between plants in the row of 0.3 m. The variety Desiree was used as a control, 10 tubers planted in the middle row (3rd) in each elementary plot.

Agrotechnical practices that are applied to the experimental field are the standard in potatoe growing technic. Before planting in the spring and before harvesting the tubers in autumn of 2014 from each elementary plot samples of soil were taken to determine the presence, abundance and vitality of *Globodera rostochiensis* cysts. In the phase of intensive bulking of potato tubers 16.07.2014 sampling was carried out again, taking 10 plants - one of each variety tested, to determine the presence of cysts on the root system. From the root zone of sampled plants soil samples were collected (0.5 kg) in order to determine number of males in the root zone.

RESULTS

Examination of the soil samples from the elementary plot, taken immediately before planting, established an equal number of cysts in each plot (average of 30 cysts in 500 ml of soil) with content vitality of about 50%, a sufficient inoculation potential for infecting tested varieties (Pi= 9 eggs and J2/1 ml of soil). A review of the root system (Table 2) in the phase of intensive bulking of tubers (16.07.2014) in susceptible varieties (Desiree and Lusa) massive cysts of GPCN - Ro1 developement was found. In five GPCN - Ro1 resistant varieties (Karlena, Pirol, Lady Claire, Crips4all and Arizona) cysts were not detected in the root system also no males were foundin the root zone (Table 2), which directly indicates these genotypes resistance to *Globodera rostochiensis* pathotype Ro1 populationin the locality of Planina.

Figure 1. Cysts *Globodera rostochiensis* pathotype Ro 1 on the potato root system

The analysis of soil samples ,taken from the plots after potato harvest in susceptible varieties (Desiree and Lusa), have shown high level of newly formed cysts (50 cysts/ 500 ml of soil) and almost complete emptiness of the old cysts (Pf = 30eggs and J2/1ml of soil). On the plots with all resistant varieties (Karlena, Pirol, Lady Claire, Crips4all and Arizona) analysis after harvest have not determined newly formed cysts, while the vital content of old cyst was reduced by half (50 % lower) compared to the level before planting (Pf = 4.5 eggs and J2/1 ml of soil).

Table 2. Field infected with GPCN, presence of the cysts on root system (c) and males (♂) in soil, locality of Planina in 2014.

Cultivar	Susceptibility	Cysts (c) and males (♂) on root system in soil
Karlena	R	c=0; ♂=0
Pirol	R	c=0; ♂=0
Lusa	S	c=5; ♂=5
Lady Claire	R	c=0; ♂=0
Arizona	R	c=0; ♂=0
Desiree (control)	S	c=5; ♂=5

S - susceptible cultuvar, R - resistant cultivar
Ro1 = resistance on GPCN (16. 07. 2014.)
c = cysts on root system (of 0 to 5cm in length)
♂ = males in soil (at the root zone of 0 to 5 in 100 ml of soil) (16.07.2014.)

In susceptible potato varieties a positive GPCN growth rate was detected (Pf /Pi = 3,3), while in the resistant varieties negative growth rate was detected (Pf /Pi = 0.5 eggs and J2/1 ml of soil).

DISCUSSION

Potato Cyst Nematode, both the Golden *Globodera rostochiensis* (GPCN) and the White *G.pallida* (WPCN) have become a very serious problem in potato production in countries where PCN present. Depending on the degree of PCN soil infection, production losses are movingin the range from 12-60% and some times even total. Potato is one of the most consumed food crops world-wide and in case of further spreading of PCN the food production balance would be severely affected, first locally and then worldwide. Serbi ais among the countries which have discovered PCN duringthe last 15 years. Measures that have been undertaken now are

reduced to seed potato import control and control of the presence of PCN on production areas under seed potato. This way the controls are covering about 1.000 ha per year, while large portion of potato seed production remains without control.

Potato production areas in Serbia (about 80.000 ha) remain without control of PCN, despite the fact that the EU indicated the need of gradual introduction of control of PCN on all areas where potato production exists (Poštić et al., 2013b). It is one of the requirements for export and placement of potatoes in the EU and surrounding countries. Countries which are dealing with mixed PCN population have a much difficult and more complex task in preventing their spreading and suppression. Countries with such problems are located in Western Europe and in South and Central America. Countries with just one PCN population, commonly *Globodera rostochiensis* (GPCN), are dealing with problem quite successfully (US and Canada). In the United States the area infected with GPCN - Ro1 is identified and strictly regulated varieties wise (only varieties resistant to this pathotype can be grown there). This way, in the production conditions, very successful control of the level of GPCN populations is achieved, while strict quarantine measures of prevention are exercised on imported material susceptible to WPCN or GPCN (Ro 2-5).

In England and Wales the situation is different, over the last 30 years they are dealing with an epidemic of WPCN (*Globodera pallida*), which is increasingly replacing the GPCN (*Globodera rostochiensis*). Trudgill et al. (2003) suggest that this is due to the introduction of the very commercial varieties of potatoes (Maris Piper 1966-71), and later two varieties (Cara and Pentland Juvelin), all of them resistant to GPCN, consequently in 2001 52% of the area under potato was planted with these 3 varieties. Thus inducing favorable conditions for the expansion of WPCN, which is more aggressive. The system of control measures is very complex because there are 3 pathotypes of this kind and on the other side a limited assortment of commercial potato varieties, carrying one or more genes resistant to Pa1-3. Regardless of the pathotype, GPCN or WPCN, resistance of varieties is decreasing and even vanishing, thus a fresh start is needed in the selection of new PCN resistant varieties. Genes carriers of potato resistance to GPCN are H1, K1, Fa and Fb, and to the WPCN H2 and H3 (Phillips, 1994).

For the production purposes it is desirable to maintain the initial level of the population (Pi) under Pi=2 eggs and J2/1 ml of soil (Brodie, 1996). If Pi= 0.1-1.0 eggs and J2/1 ml of soil than resistant varieties can be grown every 3rd year.

It is considered that introduction of PCN to Serbia was some 40 years ago, on small areas, spreading passively (mostly through seed potatoes). Strict control of the import, production and movement of seed potatoes is therefore required, as well as control of the presence of PCN in the areas where potatoes should be grown (both seed and commercial). Area intended for the production of seed potatoes must not be infected with PCN, while soil intended for the production of marketable potatoes should be examined for the presence of PCN, in order for the pathotype to be determined (Poštić et al., 2013b). Areas tested should be planted with PCN resistant potato varieties according to certain pathotype of PCN detected, taking into account the initial level of population (Pi), which should be below 0.2 eggs and J2 in 1 ml of soil (Brodie, 1996).

According to our previous researches in the Western Serbia *Globodera rostochiensis* pathotype Ro1 is commonly present, and there are great deal of commercially-attractive potato varieties resistant to this PCN, which can be used in order to successfully control the presence of GPCN-Ro1 (Krnjaić and Poštić, 2009; Bačić, 2010; Poštić et al., 2013b). On the other sites it is necessary first to identify the species of PCN (in the case of one species), or a mixed populations. After that determination of pathotype of PCN is required. Next according tot he obtained findings antinematode measures should be implemented and implementation control carried out until the disappearance of the infection.

On the plots under resistant varieties (Karlena, Pirol, Lady Claire, Crips4all and Arizona) after harvesting tubers in autumn there were no newly formed cysts, while the vital content of the old cyst was reduced to half (50% lower), compared to the level before planting. These results are consistent with other results (Zakabunina, 2000; Poštić et al., 2013b). Furthermore susceptible potato varieties positive growth rate of GPCN was measured (Pf /Pi=3.1), while the resistant varieties were recording negative growth rate (Pf /Pi=0.5 eggs and J21 ml of soil).

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INFLUENCE OF SUSCEPTIBLE AND TOLERANT VARIETIES ON POPULATION DENSITY OF SUGAR BEET CYST NEMATODE (*Heterodera schachtii*)

Jasmina Bačić

Agricultural Extension Service Institute Tamiš, Novoseljanski put 33, 26000 Pančevo, Serbia
E-mail: jasmina.bacic@jasminabacic.rs

ABSTRACT

The influence of susceptible and tolerant sugar beet varieties on initial (*Pi*) and final (*Pf*) population density of sugar beet cyst nematode (*Heterodera schachtii*) was investigated in Serbian northern province of Vojvodina on naturally nematode infested field in Vojka, in district of Srem during the year 2013. The susceptible (Original) and tolerant (Fiorenza) varieties, recommended as standard in testing of new nematode sugar beet varieties by Serbian Plant Protection Directorate, were planted on experimental plots of field where sugar beet was grown in period of 2011-2012. The results of this field trial indicated that the average reproductive rate of nematodes (*Pf/Pi*) in soil was slightly lower for tolerant variety (3.9) than compared to susceptible one (4.1) on a heavily infested field. The standard tolerant variety gave significantly higher average yield (17t/ha) than susceptible variety (6.6 t/ha). These results may be used by growers from the infested regions of Vojvodina when choosing sugar beet varieties in order to reduce yield loss in their crops.

Key words: *Heterodera schachtii*, sugar beet, nematode tolerant varieties, reproductive rate

INTRODUCTION

The sugar beet cyst nematode (SBCN) *Heterodera schachtii* Schmidt is a serious pest that can significantly reduce root yield and quality of sugar beet. First discovered near Halle, Germany in 1859 by H. Schacht, it was named and described by A. Schmidt in 1871 (Harveson & Jackson, 2008). It causes severe damage to sugar beet with yield losses of up to 50 % when population densities are high (Ferreira & Boley, 1993). Affected plants usually occur in patches. Beet cyst nematode damage to the roots leads to wilting of crops similar to that seen in *Rhizomania*. The most easily recognized sign of infection is the white lemon-shaped female nematodes attached to roots from about late June, approximately 2 months after planting. These females later turn brown, forming the protective cysts which remain in the soil for several years. Two generations can be completed in a season in temperate regions, but up to five may occur during the longer, hotter growing seasons of southern European countries. The best management strategy for SBCN is rotation with non-host crops and avoidance of moving SBCN-infested soil to non-infested fields by unclean machines and other means. At present, a rotation clause in the British Sugar contract forbids the growing of sugar beet in fields where a *Beta* species (i.e. sugar beet, fodder beet or red beet) has been grown in either of the two preceding years. However, research continues into the development of nematode tolerant and resistant beet varieties and use of nematode-resistant brassicaceous (cruciferous) green-manure catch crops (Alford, 2000).

As a consequence of a narrow crop rotation, SBCN became a nematological problem in the sugar beet growing areas in the Serbian northern province of Vojvodina. The last six years of mapping *of H.schachtii* in Vojvodina indicated that this pest is the most frequent near old sugar-beet factories in districts of Bačka and

Srem (Bačić, 2013). Nematode tolerant varieties have been tested as they become commercially available during recent years in Serbia (Bačić et al, 2007). The main goal of this field experiment was to assess the reproductive rate of *H. schachtii* on susceptible and tolerant varieties and which root yield would be on heavily infested field where sugar beet was grown in the continuous period of 2011-2013.

MATERIAL AND METHODS

In 2013 one susceptible and one tolerant variety, recommended as standard in testing of new nematode tolerant varieties by Ministry of Agriculture of Serbia (Sl. Glasnik RS, 31/13), were grown on a naturally nematode infested field in Vojka in Northern province of Vojvodina. The field trial was commissioned by Serbian Plant Protection Directorate. The standard susceptible (Original SESVanderHave) and tolerant (Fiorenza KWS SAAT AG) varieties, were planted on experimental plots of area of 20 m^2 with 200 plants per plot in four replications. Soil samples for analysis of nematode density were taken from each plot after planting in April (initial population density-*Pi*) and at harvest in late September (final population density-*Pf*). One composite soil sample consisted of 20 sub-samples from different points of each plot sampled on the depth of 10 cm with a special stick-auger 1cm wide. In order to extract cysts soil samples were processed using Fenwick can (Turner, 1998). An electric device was used to crush cysts extracted from 100 cm^3 of soil. Estimation of number of eggs and second-stage juveniles (J2) in homogenized solution was done using a microscope and counting chamber (Stephani, 2006, personal communication). The weight and number of healthy roots were estimated from each plot (kg/10m^2) at harvest. The nominal reproductive rate (nematode propagation value) was calculated by dividing *Pf* by *Pi*. Data on initial and final population density, reproductive rate, yield and number of healthy roots are presented as mean (M) ± standard deviation (SD) with range between minimum and maximum values. Student's t-test was used for comparing the means of two samples at the significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

The average initial and final population density and reproductive rate of susceptible and tolerant varieties do not differ significantly (Table 1). The average initial density for susceptible variety was 265 with range between 100 and 480 eggs and J2 in 100 cm^3 of soil. The average final population density was ascertained 850 with minimum 720 and maximum 900 eggs and J2 in 100 cm^3 of soil. The average initial density for tolerant variety was 305 with range between 100 and 560, while the average final population was 740 with range between 200 and 1360 eggs and J2 in 100 cm^3 of soil. The reproductive rate of *H.schachtii* in soil was slightly higher for susceptible variety 4.1 (2.0-7.2) when compared to average *Pf*/*Pi* for tolerant variety 3.9 (0.3-8.3). The standard tolerant variety gave significantly higher average yield 17 t/ha (14.0-21.5) than non-tolerant variety 6.6 t/ha (5.0-9.5). The average number of healthy roots was significantly greater for the tolerant variety 61.5 (48-76) than for the susceptible one 43.2 (37-53).

These results showed that the standard nematode tolerant variety performed better than the standard susceptible one on highly infested field. Nematode „tolerance" concept (partial tolerance) restricts damage due to infection and tolerates the attack of the pathogen. Different kinds of sources of partial resistance were used: *Beta maritima* accessions and non-adapted germplasm while for „nematode resistant" varieties which means complete resistance *Beta procumbens* was used (Sels, 2014). With SBCN, the distinction between tolerance and resistance is particularly important. A tolerant variety will grow relatively unimpeded by the pest, but will have little impact on nematode populations.

Table 1. Average values of *Pi*, *Pf*, *Pi*/*Pf,* yield and number of healthy roots of susceptible and tolerant varieties tested on nematode-infested field in Vojka in 2013.

Parameters	Susceptible variety M ± SD (min-max)	Tolerant variety M ± SD (min-max)
Initial population density- *Pi*	265a ± 157.8 (100-480)	305a ± 230.6 (100-560)
Final population density-*Pf*	850a ± 108.9 (720-980)	740a ± 535.2 (200-1360)
Reproductive rate *(Pf/Pi)*	4.1a ± 2.2 (2.0-7.2)	3.9a ± 3.3 (0.3-8.3)
Yield (t/ha)	6.6a ± 2.0 (5.0- 9.5)	17.0b ± 3.3 (14.0 – 21.5)
Number of healthy roots	43.2a ± 6.9 (37-53)	61.5b ± 11.7 (48-76)

* Mean values marked with the same letter do not differ significantly (t-test, $p < 0.05$)

On the other hand, a resistant variety also reduces the population of nematodes, effectively cleaning the soil. Nematode propagation values of than 1 indicate an increase in population, less than 1 a decrease. Trials undertaken in Germany between 2003 and 2006 by LWK Niedersachsen showed that both susceptible and tolerant varieties will always multiply nematodes and that the multiplication factor is generally dependent on the initial population-the smaller the population, the stronger the multiplication (Syngenta, 2011).

The international economic thresholds for *H.schachtii* are different. The initial soil population density of approximately 3 eggs, range between 2 and 4 juveniles in 1 cm^3 of soil may result in yield loss (Gray et al, 1992). The field trial in Vojka showed that the average initial population densities of susceptible and tolerant varieties were at these levels (265 and 305). The recommended threshold values for use of tolerant varieties are different (Stevens, 2014). They depend on type of soil, climate, number of generation and range between 100 (Italy) and 500 (Germany). Higher temperatures in southern European countries as Italy and Serbia, encourage the rate of development of SBCN. The weather conditions in Vojka during 2013 with rainy spring and dry summer were very favourable for nematodes. In France, influence of resistant sugar beet on nematode population dynamics was investigated in 14 naturally infested fields. Results indicated that the influence on soil *H. schachtii* population densities of sugar beet cultivars, resistant or not, is always correlated with *Pi*. At low initial infestations, final population (*Pf*) are increased and at very low infestations, below 5 juveniles/g, multiplication may be high, even with a resistant cultivar. Reproductive rate was negatively correlated with *Pi* across environments of both resistant and susceptible cultivars (Caubel et al, 2000). According to Liesenfeld et al. (2012) tested tolerant varieties showed no or only small reproduction rates under high intial nematode populations. On the other hand, low initial populations could triple nematode populations.

Introduction of tolerant varieties is now in progress in many countries. According to Minerva (2014), the use of these varieties has spreaded in period of 2010-2014 from 8 to approximately 18%. Sugar yield production in Italy (Bologna) increase from 7.5-8 t/ha in the period of 2000-2004 to 9-10 t/ha in the period of 2005-2011 thanks the use of nematode tolerant varieties. According to Stewart at el. (2014), use of a tolerant variety in fields heavily infested with SBCN has increased by 15 tons per acre when compared to non-tolerant varieties. It should be noted that SBCN will develop and reproduce on these tolerant varieties but poorly. Growers should use these varieties prudently as overuse may lead to a shif resistance to SBCN. These varieties are very susceptible to *Cercospora* and *Rhizoctonia* and fungicides should be used appropriately.

The findings of this field experiment in Vojka indicate that the reproductive rate of *H. schachtii* does not differ significanly among susceptible and tolerant variety on a heavily infested field. The initial population density under 1 eggs or juveniles/cm^3 of soil is not recommended as threshold value for use of tolerant variety in this region. Although the tolerant variety gave significantly higher yield compared to susceptible one in Vojka, it was smaller than in non-infested fields in Vojvodina where the average yield of sugar beet was between 47.8 t/ha in 2013 (Anonymous, 2013). These results may be used by growers from the infested regions of Vojvodina when choosing sugar beet varieties in order to reduce yield loss in their crops. This research represents a basis for further investigation of thresholds and real levels of damage for tolerant beet varieties compared to susceptible ones in infested regions within activities of Plant Protection Forecasting and Warning Service of Vojvodina.

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HERBICIDES IN SPRING OILSEED RAPE: SOIL AND FOLIAR APPLICATION

Petar Mitrović[1], Dragana Marisavljević[2], Danijela Pavlović[2], Ana Marjanović-Jeromela[1], Željko Milovac[1] and Milan Jocković[1]

[1] Institute of Field and Vegetable Crops, Novi Sad,Serbia
[2] Institute for Plant Protection and Environment, Belgrade, Serbia
(Corresponding author: petar.mitrovic@nsseme.com)

ABSTRACT

Possibility to chemically control weeds in spring rapeseed has been tested in two locations, Novi Sad and Kragujevac. The following herbicides were tested: Trefgal (a.i. trifluralin), Gamit (a.i. clomazone), Globus (a.i. quizalofop-p-ethyl) and Lontrel 100 (a.i. clopyralid). Combinations of herbicides Trefgal + Gamit and Trefgal + Lontrel 100 showed the highest efficiency on annual broadleaf weeds in both localities. The tested herbicides had no effect on annual and perennial weed species *Agropyrum repens, Cirsium arvense, Cynodon dactylon*, as well as the *Hibiscus trionum*. The herbicide Gamit, in the combination Gamit + Trefgal, exhibited phytotoxicity to the rapeseed crops in both locations. Simultaneously we tested the effect of the herbicides on hectoliter weight of seed and oil and protein content in seed. In Kragujevac, the tested herbicides were not adequately efficient for the weeds present in that location. In Novi Sad, the location predominated by annual broadleaf weeds, the performance of these herbicides was much better. The tested herbicides differed significantly in their effect on the quantity and quality parameters of rapeseed.

Key words: spring rapeseed, weeds, herbicides

INTRODUCTION

The rapeseed (*Brassica napus* L.) belongs to the *Brassicaceae* family and it has a winter and a spring form. The spring form is mainly grown in areas with very cold winters (Kanada, Sweden). In Europe, excluding Sweden, the acreage under spring rapeseed is small (Kondić et al., 2008).

Winter rapeseed cultivars are dominant in European countries (Marinković et al., 2010), but selection and breeding of spring cultivars intensified in Europe in the second half of the 20th century (Mustapić et al., 1984).

Chemical weed control is not a mandatory practice in winter rapeseed and it is performed as needed and not on the entire acreage. In our agricultural practice, the rapeseed is considered a competitive crop and herbicide use is not considered cost-effective (Mitrović et al., 2009). However, questionnaires have shown that herbicides application is practiced both, in the fall and spring (Marisavljević et al., 2007).

The spring rapeseed is less competitive against weeds than the winter rapeseed, especially at the beginning of the growing season. The optimum time for spring rapeseed planting is from mid-March to mid-April, a period that coincides with the emergence of germination and spring weeds (Konstatinović et al., 2007). If seedbed preparation is performed well and the spring rapeseed is planted at optimum date, the crop usually stays weed-free in the early stages of development. However, post-sowing emergence of weed species (15 to 20 days after sowing) can cause reductions in oil yield and quality (Klaaβen, 2006). The same author recommends that, in addition to chemical treatment before or after sowing and before crop emergence, an additional treatment with metazachlor should be performed after crop emergence, to eradicate the weed that emerged in the meantime. Davies (2005) recommends a similar weed control schedule for the spring rapeseed, the difference being the pre-sowing application of trifluralin instead of clomazone and the post-emergence application of metazachlor and clopyralid.

In the period after emergence of spring rapeseed, broadleaf weeds are dominant in rape plots, while grassy weeds prefer wet and neglected plots (Gunsolus and Oelke, 2000; loc. cit. Konstantinović et al., 2007). Particularly harmful are species from the *Brassicaceae* family (*Sinapis arvensis, Raphanus raphanistrum, Thlaspi arvense, Diplotaxis muralis*) because they develop faster than the crop, shade and smother it. They occur in seed plots too. In practice there is no suitable herbicide for their control, so it is necessary to apply mechanical measures and to treat chemically the previous crop (Konstantinović et al., 2007; Klaaβen, 2006). Large temperature variations in April and the first half of May tend to slow down the growth of rapeseed which results in intensive weed occurrence in the early stages of crops development (Brennan and Thill, 1993). Many weeds, especially those from the *Brassicaceae* family, can in addition to direct damage also cause indirect damage as vectors of harmful fungi and insects. For example, a weed species *Capsella bursa pastoris* is host to a parasitic fungus *Albugo candida* (Leino, 2006; Antonijević and Mitrović, 2007). Harvest of weed-infested crop produces rape seed with admixtures of weed seeds which increase the cost of drying and reduce the quality of oil and proteins (Klaaβen, 2006; Davies, 1999). The above data indicate that it is important to control weeds in spring rapeseed.

The objective of this study was to investigate the possibility of controlling weeds in rapeseed plots and to assess the impact of herbicides on yield and quality parameters of spring rapeseed.

MATERIAL AND METHODS

This study was carried out in 2009 in two locations, Kragujevac and Novi Sad, using the standard method for testing the efficiency of herbicides in rapeseed crops (Anon, 2004). Material for the experiment was spring rapeseed cultivar Jovana, in property of Institute of field and vegetable crops from Novi Sad, Serbia, registered for commercial use. The experiment was set up as a randomized block design with three replicates. Plot size was 30m^2. Basic data for the experiment are shown in tables 1 and 2.

Chemical treatment was performed by means of a backpack sprayer "Solo", with an extension tube fitted

Table 1. Basic data for the experiment

Location	Kragujevac	Novi Sad
Soil type	Pseudogley (parapodzol)	Degraded chernozem
Previous crop	Wheat	Seed pea
Planting date	10 Apr 2009	25 Mar 2009
Application date	08 Apr 2009 Trefgal and Gamit Incorporated in soil	23 Mar 2009 Trefgal and Gamit
Assessment dates	20 May 2009 Lontrel 100 and Globus 1st assessment: 10Jun 2009 2nd assessment: 10 Jul 2009	18 May 2009 Lontrel 100 and Globus 1st assessment: 26 May 2009 2nd assessment: 10 Jul 2009
Harvest	10 Aug 2009	04 Aug 2009

Table 2. Herbicide variants tested

Treatment	Herbicide (active substance)	Dose per ha	Application time
1.	Control	-	-
2.	Trefgal (trifluralin 480 g/l)	2.5 l/ha	Pre-plant – incorporation
3.	Trefgal (trifluralin 480 g/l)+ Lontrel 100 (clopyralid 100 g/l)	1.5 l/ha + 1.0 l/ha	Pre-plant – incorporation + post-emergence and after weed emergence
4.	Trefgal (trifluralin 480 g/l)+ Gamit (clomazone 480 g/l)	1.5 l/ha + 0.2 l /ha	Pre-plant – incorporation
5.	Gamit (clomazone 480 g/l)	0.2 l/ha	Pre-plant – incorporation
6.	Gamit (clomazone 480 g/l)	0.3 l/ha	Pre-plant – incorporation
7.	Lontrel 100 (clopyralid 100 g/l) + Globus (quizalofop-p-ethyl 50 g/l)	1.0 l/ha + 2.0 l/ha	Post-emergence and after weed emergence
8.	Globus (quizalofop -p-ethyl 50 g/l)	2.0 l/ha	Post-emergence and after weed emergence
9.	Control with hoeing	-	-

with eight Lurmark 03 F 110 nozzles. Herbicides were mixed with water, which was applied at a rate of 300 l/ha when rapeseed plants were 10 cm tall and weeds in stage of 2-6 pairs of leaves. In addition to the tested herbicides, the experiment included also two controls (with hoeing and without hoeing).

The effectiveness of the herbicides was assessed by counting weed plants (number of weeds/m^2). Herbicides phytotoxicity for rapeseed was estimated at the time of herbicide efficiency assessment, visually, on the EWRS scale 1-9: 1 - healthy plants with no symptoms, 2 - slight phytotoxic symptoms, 3 - medium, but clearly recognizable symptoms, 4 - pronounced symptoms whose effect on yield is uncertain, 5 - strong symptoms, growth disorder, chlorosis perceivable, etc., when yield reduction is expected to occur, 6, 7, 8, 9 - severe damage to complete destruction of plants (Anon, 1981). Foliar application of Lontrel 100 and Globus was made on 20 May 2009, when rapeseed plants were about 10 cm tall and most of the weeds were at the stage of 2-6 developed leaves (at the time of treatment, weed infestation rate was not assessed). Rapeseed yield and quality were determined by measuring and analyzing the following parameters: grain yield (kg/30 m^2), hectoliter weight, oil content (%) and protein content (%) in seed. Basic statistical calculations of rapeseed yield and quality were done by the t-test (Mead et al., 1996).

First assessments of weed infestation rate in the crop were done two months after planting, at both sites (Tab. 1). For foliar treatment, assessments were done 30 days after planting. The reason for a rather late performance of the assessments were poor weather conditions (a spell of extreme drought). In Novi Sad, a total rainfall from the beginning of April till mid-May was 15 l/m^2, with temperatures soaring up to 30°C in the first half of May. The experiment in Novi Sad was sprinkler irrigated on 10 April to provoke the emergence of rapeseed plants and weeds. Similar weather conditions were registered in the second location, except for a 30 l/m^2 rainfall at the beginning of May.

RESULTS AND DISCUSSION

Tables 3, 4 and 5 show the results of the first and second assessments of weed infestation (number of weeds/m^2) performed in the locations of Kragujevac and Novi Sad. Data shown in tables 3 and 4 indicate that 15 weed species were present in the location of Kragujevac, 3 grassy (2 perennials and 1 annual) and 12 broadleaf weeds (2 perennials and 10 annuals). *Hibiscus trionum*, *Cynodon dactylon* and *Agropyrum repens* were dominant weeds in the experiment at the time of both assessments in the location Kragujevac. At the time of application of Trefgal and Gamit, which were incorporated on 8 April 2009, the soil was relatively favorably humid and the first substantial rain fell on 30 April 2009 (about 30 l/m^2), which has not reduced the effect of these herbicides.

Table 3. Weed infestation rate in rapeseed crop, Kragujevac location, 1st assessment, 10 Jun 2009

Weeds	Treatment							
	1	2	3	4	5	6	7	8
	number of weeds/m^2							
Agropyrum repens	5	0	6	0	5	2	0	1
Amaranthus retroflexus	3	0	0	0	2	0	0	2
Atriplex patula	2	0	0	0	0	0	0	0
Chenopodium album	4	1	0	1.5	2	2	0	6
Cirsium arvense	0	4	2	0	0	0	0	0
Cynodon dactylon	10	9	11	0	8	0	0	5
Echinocloa crus -galli	4	1	2	2	0	3.5	0	0
Hibiscus trionum	4	4.5*	7	8	6*	5*	8	11
Linaria vulgaris	1	0	0	1	1	0	0	1
Matricaria chamomilla	4	0	0	0	0	0	0	1
Polygonum lapathifolium	3	0	0	3.5	2.5	2	3.5	1.5
Polygonum convolvulus	3	2	0	2	3	2	3	3
Rubus caesius	1	0	0	0.5	0	1	0	1
Vicia craca	1	0	0	0	0	0	0	1
Xanthium strumarium	1	1	0.5*	1	1.5*	1.5*	0	1
Phytotoxicity	-	2	2	2-3	2-3	3-4	2	2

*plants with arrested growth but not destroyed, fhytotoxicity assessment (1-9)

All of the tested herbicides showed some effect on the weeds present in Kragujevac, however, because of the presence of grassy weeds and an increased number of plants of *Hibiscus trionum*, which was not effectively controlled by the application, the overall effect of herbicides was unsatisfactory. If total number of weeds/m^2 is taken as a parameter of efficiency, then the following herbicide combinations were most effective in this location: Trefgal + Gamit (1.5 l/ha + 0.2 l/ha) and Trefgal + Lontrel 100 (1.5 l/ha + 1.0 l/ha) but this conclusion can not be considered as fully reliable. The reason for doubts is a highly uneven distribution of weeds in the trial, where individual plots were under great pressure of *Agropyrum repens*, *Cynodon dactylon, Cirsium arvense*, *Rubus caesius* and a parasitic angiosperm *Cuscuta campestris*, which parasitized both weeds and rapeseed plants.

The herbicide Gamit, in the combination Gamit + Trefgal, exhibited phytotoxicity to the rapeseed crops in both locations. The rapeseed plants treated with the combination Trefgal and Gamit in the amounts of 1.5 l/ha + 0.2 l/ha exhibited low phytotoxicity which was manifested as etiolation of individual leaves totalling about 10% of the plant foliage at the stage of 1-3 true leaves. The plants treated with Gamit alone, in the amount of 0.2 l/ha, exhibited similar symptoms. Gamit applied in the quantity 0.3 l/ha caused somewhat more pronounced symptoms, etiolating about 20% of the plants at the stage of 1-3 true leaves. These symptoms are known to occur in response to the application of clomazone based herbicides, and they are temporary and disappear in the course of further plant growth. The phenomenon was discussed by Davies (2005).

In the location of Novi Sad, 15 weed species were registered, one grassy (perennial) and 14 broadleaf weeds (2 perennials and 12 annuals). Regardless of a similar number of weed species as in the Kragujevac experiment, the number of weeds was significantly lower, ranging from 2 to 10 weeds/m^2. In both locations, the combinations Trefgal + Gamit (1.5 l/ha + 0.2 l/ha) and Trefgal + Lontrel 100 (1.5 l/ha + 1.0 l/ha) were most effective in weed control. In this experiment too, certain weeds were unevenly distributed (in patches or as individual plants). The effective performance of pre-emergence application timings ofherbicide was observed on reduced growth and population of weeds from the very beginning, which increased seed yield in rapeseed significantly. Similar result has been reported by Khan and Mumtaz (1995), Yadav et al. (2004. and Singh et al. (2001). Application of herbicides decreased the weed density over control. Effectiveness of herbicides in controlling weeds has been reported by Yadav et al. (2004). Bagherani and Shimi (2002) have also reported that among five herbicides (trifluralin, ethalfluralin, cyanazine, alachlor and propyzamide), the most efficient treatment was trifluralin. In order to determine as precisely as possible the impact

Table 4. Weed infestation rate in rapeseed crop, Kragujevac location, 2nd assessment, 10 Jul 2009

Weeds	Treatment							
	1	2	3	4	5	6	7	8
	number of weeds/m^2							
Agropyrum repens	5	0	6	0	5	2	0	1
Amaranthus retroflexus	3	1	1	0	2	0	1	2
Atriplex patula	2	0	0	0	0	0	0	1
Chenopodium album	4	2	3	1.5	4.5	1	2	6
Cirsium arvense	3	3.5	2	1	0	0	0	0
Cynodon dactylon	13	11	14	0	10	0	0	7
Echinocloa crus -galli	4	1	2	2	1	0	0	0
Hibiscus trionum	10	8*	7	8*	6*	5*	8	11
Linaria vulgaris	1	0	0	1	1	0	0	1
Matricaria chamomilla	4	0	0	0	0	0	0	1
Polygonum lapathifolium	3	2	0	4	3.5	2	4	2
Convolvulus arvensis	3	2	0	2	3	2	3	3
Rubus caesius	1	0	0	1	0.5	1	0	1
Vicia craca	1	0	0	0	0	0	0	1
Xanthium strumarium	2	2.5	0	2	2.5*	2*	0.5	1
Phytotoxicity		2	2	2	2	2	2	2

*plants with arrested growth but not destroyed, phytotoxicity assessment (1-9)

of the tested herbicides on the weeds and the rapeseeds, the rapeseed crops were harvested in both locations and experimental units were measured for yield, hectoliter weight, oil content and protein content.

The results for seed yield showed that, on average, the yields were higher in the location of Novi Sad than in the location of Kragujevac. This result is of expected since the number of weeds was significantly higher in Kragujevac than in Novi Sad, i.e., the crop generally had worse conditions for growth and development in the former location.

In addition to the impact of weeds, crop yield was also affected by soil type. The pseudogley - parapodzol soil in Kragujevac is inferior to the degraded chernozem soil in Novi Sad. Finally, the previous crop in Novi Sad was seed pea while in Kragujevac it was wheat. In Kragujevac, the yield in the control variant without hoeing varied from 0.420 kg/30m^2 to 1.120 kg/30m^2, and in the hoed control from 1.020 kg/30m^2 to 1.320 kg/30m^2. In the herbicide-treated plots, the yield ranged from 0.340 kg/30m^2 to 2.120 kg/30m^2. In the location of Novi Sad, the control variant without hoeing yielded from 1.620 kg/30m^2 to 1.920 kg/30m^2, while the hoed control yielded from 1.440 kg/30m^2 to 2.660 kg/30m^2. In the herbicide-treated plots, the yield of the tested varieties ranged from 1.320 kg/30m^2 to 2.580 kg/30m^2. T-test results for this parameter (Tab. 6) showed that differences between the values of yield between control and treatments as well as between different treatments were not statistically significant. The large yield differences in the same location were due to uneven distribution of weeds. In some plots, the crop was almost completely destroyed because the weeds, present in large numbers, were not effectively controlled by the applied herbicide. In other plots treated with the same herbicide, weed infestation was low and crop yield was much higher.

The second analyzed parameter was the hectoliter weight of seed which ranged from 155.0 g to 169.5 g in the location of Kragujevac and 156.0 g to 167.0 g in the location of Novi Sad. In Kragujevac, statistically significant differences for this parameter were shown between the hoed control and the treatment 8 (Globus 2.0 l/ha) and between the treatments Trefgal 2.5 l/ha and Lontrel 100 1.0 l/ha + Globus 2.0 l/ha. The oil content in seed ranged from 35.93% to 40.39% in the location of Novi Sad, which was lower than the oil content in the location of Kragujevac, which ranged from 35.95% to 45.19%. Statistically significant differences in this parameter were recorded between the control variants (with and without hoeing) and the variants Trefgal 1.5 l/ha + Lontrel 100 1 l/ha and Gamit 0.3 l/ha in Novi Sad and between the control variants (with and without hoeing) and the variant Globus 2.0 l/ha in Kragujevac. The plants treated with the same herbicide variants showed no statistically significant differences.

Table 5. Weed infestation rate in rapeseed crop, Novi Sad location, 1st assessment, 6 May 2009

Weeds	Treatments							
	1	2	3	4	5	6	7	8
	number of weeds/m^2							
Ambrosia arthemisiifolia	0	0	0	1	0	0	0	1
Amaranthus retroflexus	3	0	2	0	0	0	4	3
Bromus mollis	1	0	0	0	0	0	0	0
Chenopodium album	3	0	0	0	1	0	2	3
Capsella bursa pastoris	0	0	0	0	0	0	0	0.5
Cirsium arvense	1	0	0	0	0	0	0	0
Convolvulus arvensis	1.5	1	1	0	1	3.5	0	0
Chenopodium hybridum	4	1	1	0	0	0	1	2
Euphorbia helioscopia	0	0	0	0	1	0	0	0
Fumaria officinalis	0	0	0	1	0	0	0	1
Lactuca serriola	1	1	1	0	0.5	3	1	0
Sinapis arvensis	0	0	0	0.5	0	0	0	2
Solanum nigrum	2	1	1	1	2	2.5	0	1
Sonchus arvensis	1	0.5	0	0	0	0	0	0
Xanthium strumarium	0	0	1	0	0	0	0	0
Phytotoxicity		2	2	2-3	2-3	3-4	2	2

*plants with arrested growth but not destroyed, phytotoxicity assessment (1-9)

Table 6. Statistical significance of differences in rapeseed yield and quality parameters between the treated variants and the untreated control variants, in the two locations

			Treatments						
			2	3	4	5	6	7	8
Yield kg/30m^2	Loc. I	KBK	ns	ns	ns	ns	0.025*	ns	ns
		KSK	ns	ns	ns	ns	0.035*	ns	ns
	Loc. II	KBK	ns	ns	ns	ns	0.053*	ns	ns
		KSK	ns	ns	ns	ns	0.027*	ns	ns
Hectoliter weight (g)	Loc. I	KBK	ns	ns	ns	ns	ns	ns	ns
		KSK	ns	ns	ns	ns	ns	ns	ns
	Loc. II	KBK	ns	ns	ns	ns	ns	ns	ns
		KSK	ns	ns	ns	ns	ns	ns	0.039*
Oil content (%)	Loc. I	KBK	ns	0.003**	ns	ns	0.021*	ns	ns
		KSK	ns	0.015*	ns	ns	0.034*	ns	ns
	Loc. II	KBK	ns	ns	ns	ns	ns	ns	ns
		KSK	ns	ns	ns	ns	ns	ns	0.032*
Protein content (%)	Loc. I	KBK	0.04*	ns	ns	ns	ns	ns	0.024*
		KSK	0.009**	ns	ns	ns	ns	ns	ns
	Loc. II	KBK	ns	ns	ns	ns	ns	ns	ns
		KSK	ns	ns	ns	ns	ns	ns	ns

P<0.01 **; P<0.05 *; KBK- control; KSK- control with manual hoeing; Loc. I – Novi Sad; Loc. II – Kragujevac; NS-differences not significant

The protein content in seed ranged from 18.83% to 27.08% in the location of Kragujevac and 26.28% to 28.08% in the location of Novi Sad. In the location of Novi Sad, statistically significant differences in the values of this parameter were found between the controls and the variant Trefgal 2.5 l/ha, and the control without hoeing and the variant Globus 2.0 l/ha. A statistically significant difference between the treatments was observed only in Novi Sad, comparing the variants Trefgal 2.5 l/ha and Gamit 0.3 l/ha.

CONCLUSION

Neither herbicide tested in the location of Kragujevac was sufficiently efficient in controlling the dominant weed species in the experiment (*Hibiscus trionum*, *Cynodon dactylon, Agropyrum repens, Cirsium arvense* and *Rubus caesius*). The tested herbicides exhibited a certain measure of efficiency in the control of annual grasses and broadleaf weeds at this site. In plots dominated by these weeds, best results were obtained with the combinations Trefgal + Gamit (1.5 l/ha + 0.2 l/ha) and Trefgal + Lontrel 100 (1.5 l/ha + 1.0 l/ha). The tested herbicides showed higher efficiency in the location of Novi Sad. Best effects were demonstrated by the combinations Trefgal + Gamit (1.5 l/ha + 0.2 l/ha) and Trefgal + Lontrel 100 (1.5 l/ha + 1.0 l/ha). The tested herbicides showed no adverse effect on the yield and hectoliter weight of seed in either location, with the exception of Globus in Kragujevac, which affected the control variants. Oil content was negatively affected by the combination Trefgal + Lontrel 100 (1.5 l/ha + 1.0 l/ha) in the location of Novi Sad and by Globus (2.0 l/ha) in the location of Kragujevac. Trefgal (2.5 l/ha) and Globus (2.0 l/ha) exhibited a negative effect on protein content in the location of Novi Sad, while there were no statistically significant negative effects in the other location. The differences in the oil and protein contents in seed observed between the two locations were due to the effects of climatic and edaphic factors, not of the tested herbicides.

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НОВАЯ ПРЕПАРАТИВНАЯ ФОРМА ГЕРБИЦИДОВ ДЛЯ ЗАЩИТЫ САХАРНОЙ СВЕКЛЫ

Салис Каракотов Добаевич[1], Елена Желтова Владимировна[1], Артем Голубев Сергеевич[2] и Татьяна Маханькова Андреевна[2]

[1]ЗАО «Щелково Агрохим»
Россия, г. Щелково Московской обл., ул. Заводская, д. 2, zheltova@betaren.ru
[2]Государственное научное учреждение Всероссийский научно-исследовательский институт защиты растений
Россия, г. Санкт-Петербург, Пушкин, шоссе Подбельского, 3 golubev100@mail.ru

АБСТРАКТ

Защита сахарной свеклы от сорняков в Российской Федерации предусматривает внесение гербицидов, в том числе препаратов на основе десмедифама и фенмедифама. Использование инновационных подходов в создании препаративных форм позволило фирме «Щелково Агрохим», одному из крупнейших производителей пестицидов в России, разработать гербицид Бетарен 22 (110 г/л десмедифама + 110 г/л фенмедифама), выпускающийся в форме масляного концентрата эмульсии (МКЭ). Эта препаративная форма обеспечивает значительное улучшение поглощения гербицида. Для изучения действия гербицида в форме масляного концентрата эмульсии в полевых условиях были поставлены мелкоделяночные опыты. Гербицид Бетарен 22, МКЭ вносили в уменьшенных по действующему веществу (по сравнению с эталоном) нормах применения.

В Рязанской области и Краснодарском крае биологическая и хозяйственная эффективность гербицида Бетарен 22, МКЭ была на уровне эффективности эталона в форме концентрата эмульсии, а в Волгоградской области превосходила ее, в то время как доза действующего вещества была снижена более, чем на 30%. В целом, уровень действия обоих препаратов был приблизительно одинаков, что подтверждает справедливость выдвинутой нами гипотезы о большей эффективности гербицида в форме масляного концентрата эмульсии по сравнению с аналогичным гербицидом в форме концентрата эмульсии.

Учитывая, что в сельскохозяйственном производстве посевы свеклы сахарной, как правило, обрабатываются баковыми смесями гербицидов с разным спектром действия (например, сульфонилмочевинами, клопиралидом, граминицидами), способных увеличить силу действия первых, показанная препаратом эффективность является достаточной для рекомендации его производству. Создание новой масляной формуляции карбаматных гербицидов позволило снизить гербицидную нагрузку при сохранении биологической эффективности. Наиболее ярко данный эффект проявляется в жарких и засушливых условиях.

Ключевые слова: Сахарная свекла, гербициды, препаративные формы

ВВЕДЕНИЕ

Технология выращивания свеклы сахарной предусматривает проведение обязательных мероприятий по борьбе с сорными растениями. Широкую известность у производственников получили гербициды на основе десмедифама и фенмедифама (Дворянкин, 2005).

Эти действующие вещества ингибируют фотосинтез, фиксацию растениями углекислоты, угнетают процесс фосфорилирования, вызывая тем самым нарушения энергетического баланса и основных

метаболических реакций. Фенмедифам и десмедифам проникают через листовую поверхность и обладают трансламинарным действием [с сайта http://www.betaren.ru/rus/preparaty/gerbicidy1/betaren_22_mke/]- (2).

В настоящий момент в РФ зарегистрировано более двух десятков гербицидов на основе этих действующих веществ. Подавляющее большинство из этих препаратов выпускается в форме концентрата эмульсии (КЭ) и содержит в своем составе 160 г/л десмедифама + 160 г/л фенмедифама. Фирмой «Щелково Агрохим» был разработан гербицид Бетарен 22, содержащий в своем составе 110 г/л десмедифама + 110 г/л фенмедифама, и выпускающийся в форме масляного концентрата эмульсии (МКЭ).

«Щелково Агрохим» начало разработки новых препаративных форм задолго до актуализации нанонауки в России и сейчас имеет возможность выводить на рынок препараты с уникальными свойствами, позволяющие в максимальной степени использовать целевые свойства известных действующих веществ (Каракотов, 2011).

Препаративная форма гербицида Бетарен 22, МКЭ обеспечивает значительное улучшение поглощения препарата. Масло служит проводником действующего вещества через восковый слой листа и способствует быстрому и легкому проникновению препарата глубоко в ткани вредных объектов. Использование масляных компонентов при создании формуляции требует применение высоких доз поверхностно-активных веществ, что положительно влияет на снижение поверхностного натяжения рабочей жидкости, приводит к образованию мелкодисперсной эмульсии и способствует увеличению биологической активности. Попадая на сорное растение, масляная эмульсия равномерно распределяется, образуя пленку на поверхности листа, которая препятствует испарению, кристаллизации и смыванию гербицида. Тем самым дольше сохраняется гербицидная активность препарата, не зависящая от погодных условий. Кроме того, уменьшается токсическое и фитотоксичесое действие препарата благодаря замене более токсичных компонентов препаративной формы маслом.

Целью проводимых нами исследований была проверка гипотезы о лучшей эффективности гербицида в форме масляного концентрата эмульсии по сравнению с аналогичным гербицидом в форме концентрата эмульсии.

МАТЕРИАЛЫ И МЕТОДЫ

Для достижения цели, мы поставили перед собой задачу - изучить биологическую эффективность и безопасность применения гербицида Бетарен 22, МКЭ на посевах сахарной свеклы в различных почвенно-климатических зонах РФ. Опыты с гербицидом Бетарен 22, МКЭ проводились в 2012 и 2013 годах в Рязанской области (1 почвенно-климатическая зона), Краснодарском крае (2 зона) и Волгоградской области (3 зона).

Опыты закладывались и проводились в соответствии с «Методическими указаниями по полевому испытанию гербицидов в растениеводстве» (М., 1981). Посевы свеклы осуществлялись районированными для каждой зоны гибридами (Океан, Крокодил, Пилот). Технологии их возделывания были общепринятыми для данной культуры в каждой из зон. Устранение нецелевых вредных объектов (однодольных сорняков) осуществляли путем проведения фоновых обработок противозлаковыми гербицидами (флуазифоп-П-этил, клетодим). Площадь делянок составляла от 25 до 50 $м^2$. Расход рабочей жидкости производили из расчета 200-250 л/га. Обработку проводили ручными ранцевыми опрыскивателями («Агротоп», «Резистент 3610»).

Схема опыта предусматривала одно-, двух- и трехкратное внесение испытываемого гербицида (соответственно волнам сорняков) и эталона. В качестве эталона был выбран препарат, который в своем составе большее количество обоих действующих веществ (160 г/л десмедифама и 160 г/л фенмедифама). Нормы применения обоих препаратов составляли 3,0 л/га (при однократном внесении); 1,5 л/га + 1,5 л/га (при двукратном внесении) и 1,0 л/га + 1,0 л/га + 1,0 л/га (при трехкратном внесении). Таким образом, предполагалось сопоставить эффективность препаративных форм при одинаковых нормах применения препаратов (и сниженных на 31,25% в пересчете на д.в. нормах применения испытываемого гербицида).

Учеты проводили перед проведением обработки, через 30 и 45 после нее, и перед уборкой урожая сахарной свеклы. При проведении первого и последнего учетов подсчитывали количество каждого из видов сорных растений; при проведении второго и третьего учетов, определяли также общую массу сорняков. Эффективность рассчитывали, сравнивая полученные значения со значениями в контроле без обработки.

Урожай учитывали вручную, с каждой делянки опыта. Кроме того, в течение всего периода проведения опыта наблюдали за состоянием растений сахарной свеклы на предмет возможной фитотоксичности гербицидов.

РЕЗУЛЬТАТЫ

Сорные растения, встречавшиеся в период проведения опытов в посевах сахарной свеклы, относились к малолетним двудольным растениям, которые являются целевыми объектами для гербицидов на основе десмедифама и фенмедифама. На опытных участках были распространены следующие виды сорняков: щирица запрокинутая (Amaranthus retroflexus L.), щирица жминдолистная (Amaranthus blitoides S. Wats.), марь белая (Chenopodium album L.), звездчатка средняя (Stellaria media (L.) Vill.), гречишка вьюнковая (Fallopia convolvulus (L.) A. Love), горец шероховатый (Polygonum scabrum Moench.), подмаренник цепкий (Galium aparine L.), аистник цикутовый (Erodium cicutarium (L.) L`Her.) и горчица полевая (Sinapis arvensis L.). Во время обработок сорняки находились на ранних фазах развития, наиболее уязвимых для гербицидов. Исходная засоренность посевов сахарной свеклы в опытах составляла от 47 до 74 экз./м^2 и была значительно выше ЭПВ.

Биологическая эффективность применения гербицида Бетарен 22, МКЭ была высокой во всех трех зонах проведения экспериментов.

В Рязанской области при однократном применении гербицида Бетарен 22, МКЭ снижение засоренности составляло 79-90% (рис. 1). Двух- и трехкратное внесения гербицидов были не менее эффективными - 85-90% и 83-87%. Эффективность эталона Бетан Форте, КЭ в этом регионе превышала эффективность изучаемого препарата в среднем на 2,9%, что находится в пределах допустимых отклонений.

В Краснодарском крае также препарат в виде масляной эмульсии показал высокую эффективность, статистически равную эффективности эталона при более чем на 30% более низкой дозе действующих веществ в расчете на 1 га. Следует отметить, что в этом регионе также наиболее наглядной была разница в эффективности однократного и дробного применения гербицидов.

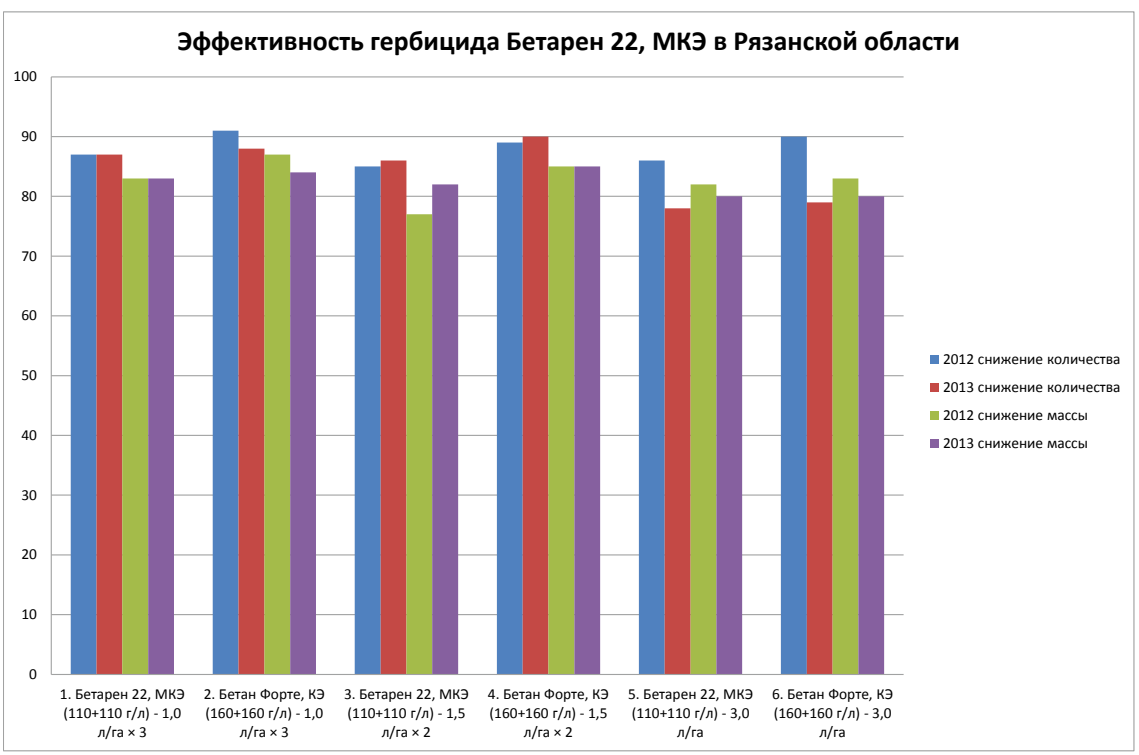

Рис. 1. Эффективность гербицида Бетарен 22, МКЭ в Рязанской области

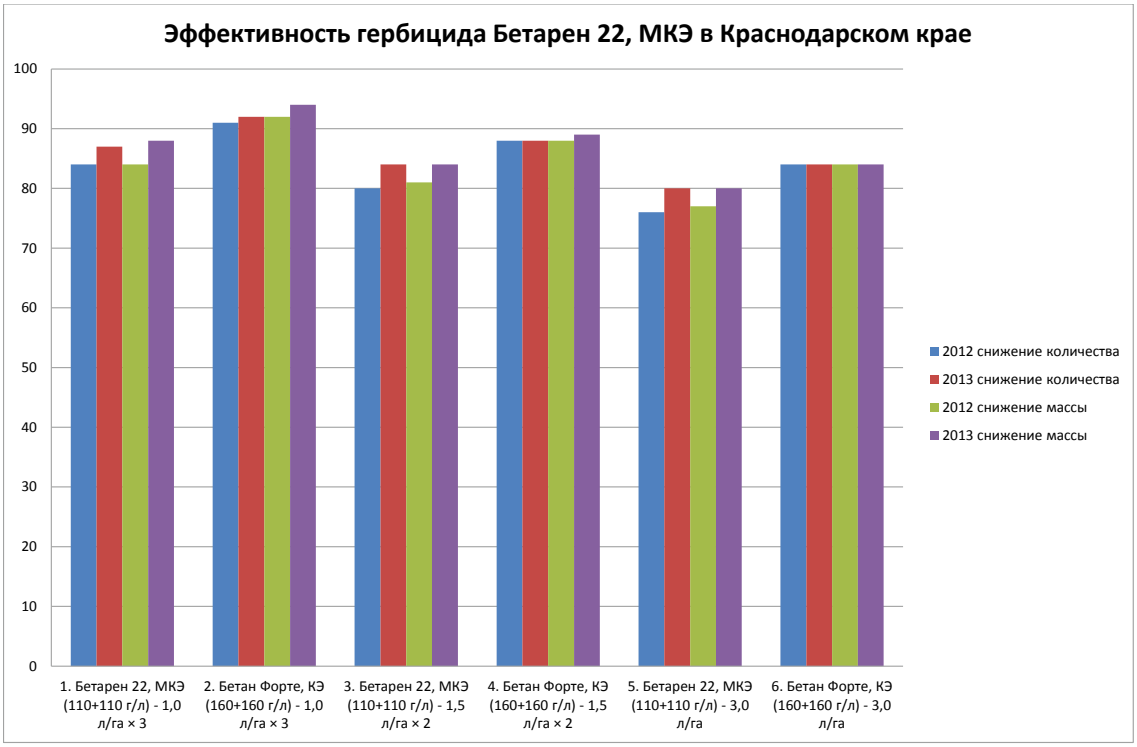

Рис. 2. Эффективность гербицида Бетарен 22, МКЭ в Краснодарском крае

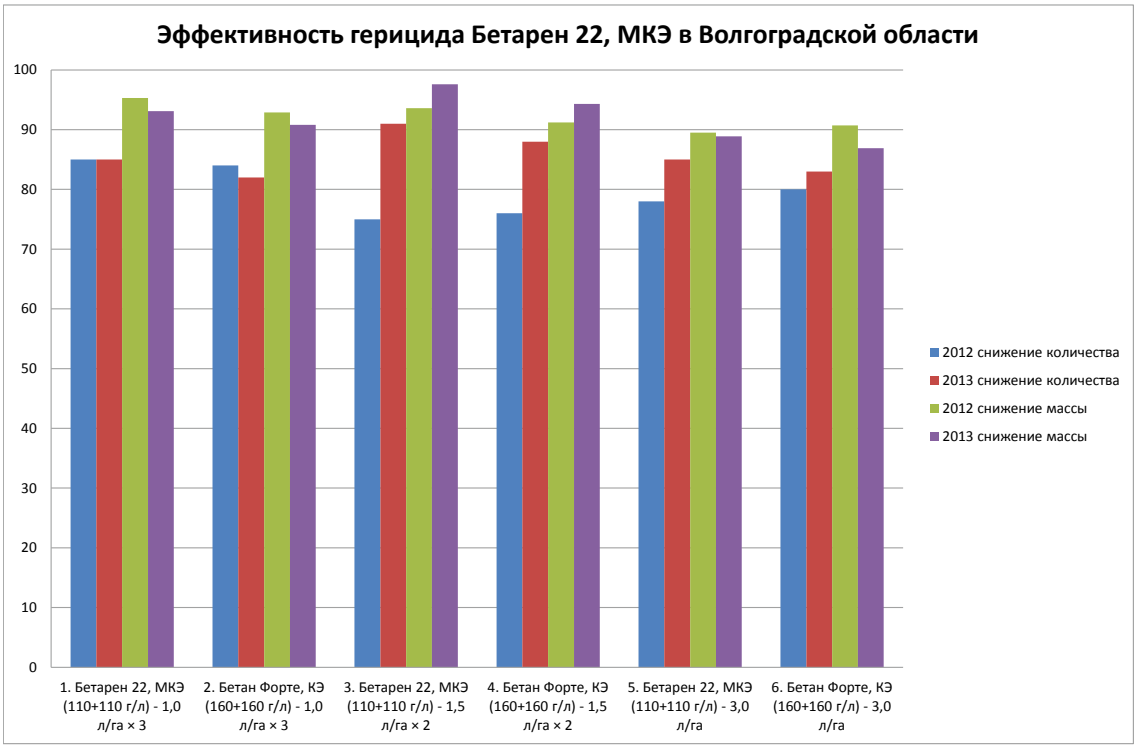

Рис. 3. Эффективность гербицида Бетарен 22, МКЭ в Волгоградской области

Волгоградская область была регионом, в котором средняя эффективность гербицида Бетарен 22, МКЭ несколько превосходила среднюю эффективность эталона Бетан Форте, КЭ (рис. 3), несмотря на 30% снижение дозы действующих веществ. Очевидно, в стрессовых условиях жаркого и засушливого климата преимущества масляной формуляции проявляются более ярко.

Следует отметить, что наименее чувствительным к гербицидам видом оказался аистник цикутовый (рис. 4). Снижение количества сорных растений этого вида при внесении препарата составляло 66-73%. Несколько более эффективным было влияние гербицидов на горец шероховатый (77-78%). Все остальные виды сорных растений были высокочувствительны (эффективность 80%) как к гербициду Бетарен 22, МКЭ, так и к эталону Бетан Форте, КЭ. Причем, изучаемый препарат был эффективнее эталона по влиянию на гречишку вьюнковую.

Наряду с биологической эффективностью препарата, важнейшее значение для агрономов имеет его хозяйственная эффективность. В наших опытах величина сохраненного урожая колебалась от 1,5 до 19,4 т/га. В Волгоградской области прибавки урожайности культуры были максимальными, в этом регионе хозяйственная эффективность изучаемого препарата превосходила эффективность эталона Бетан Форте, КЭ (что согласуется с данными по биологической эффективности).

Таблица 1. Урожайность свеклы сахарной при применении гербицида Бетарен 22, МКЭ (т/га)

Варианты опыта	Рязанская область		Краснодарский край		Волгоградская область	
	2012 г.	2013 г.	2012 г.	2013 г.	2012 г.	2013 г.
1. Бетарен 22, МКЭ - 1,0 л/га × 3	28,2	29,5	44,4	39,4	29,7	38,5
2. Бетан Форте, КЭ - 1,0 л/га × 3	30,2	31,2	45,3	40,8	29,2	38,7
3. Бетарен 22, МКЭ - 1,5 л/га × 2	28,6	29,7	44,0	39,3	28,7	39,2
4. Бетан Форте, КЭ - 1,5 л/га × 2	29,7	29,5	45,2	40,3	28,4	38,9
5. Бетарен 22, МКЭ - 3,0 л/га	27,2	29,0	43,9	39,2	25,7	39,0
6. Бетан Форте, КЭ - 3,0 л/га	28,7	29,5	44,8	40,1	25,3	38,8
7. Контроль	21,7	23,2	42,3	37,7	16,3	19,8

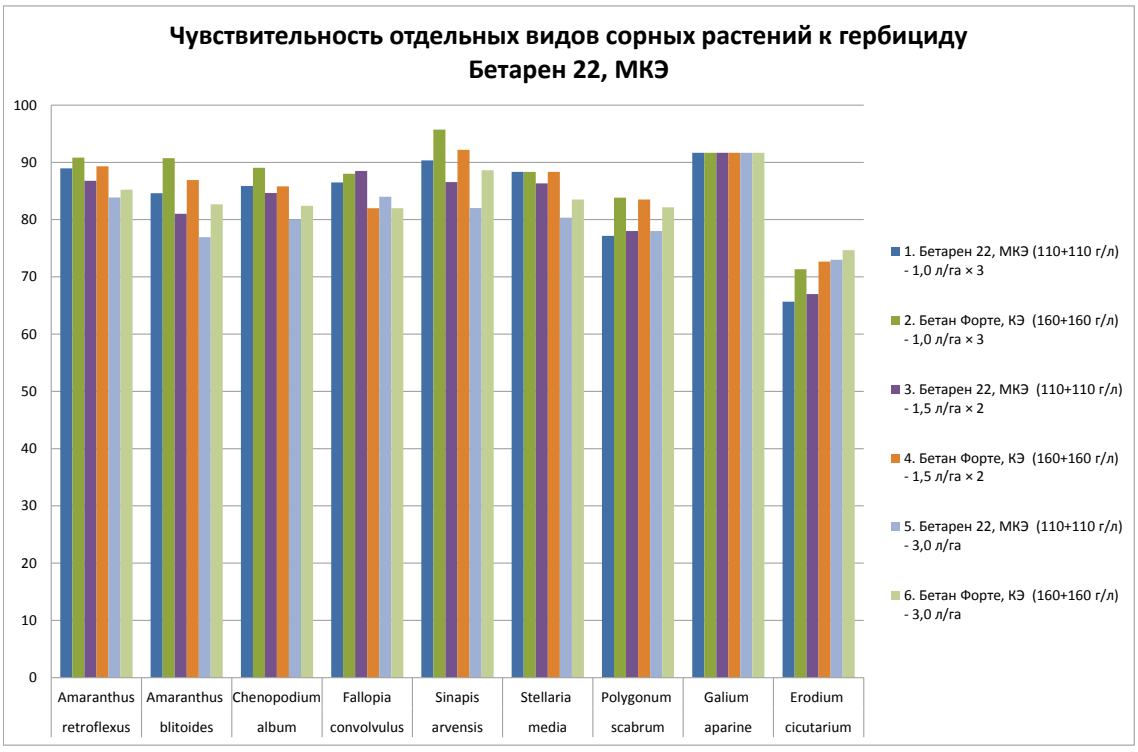

Рис. 4. Чувствительность отдельных видов сорных растений к гербициду Бетарен 22, МКЭ

В целом, уровень действия обоих препаратов был приблизительно одинаков, что подтверждает справедливость выдвинутой нами гипотезы о большей эффективности гербицида в форме масляного концентрата эмульсии по сравнению с аналогичным гербицидом в форме концентрата эмульсии.

Известно, что наиболее эффективным применение гербицидов на сахарной свекле оказывается при использовании баковых смесей бетанальных гербицидов с сульфонилмочевинами, противозлаковыми препаратами и т.д. (Берназ, Дунаева, 2008; Иващенко, 2005; Соловьев, Гераськин, 2011). В расчете на такой способ применения показанная изучаемым препаратом эффективность является достаточной для рекомендации препарата производству. При использовании препарата Бетарен 22, МКЭ будет достигаться снижение пестицидной нагрузки по действующему веществу, что благоприятно скажется на экологической обстановке.

БЛАГОДАРНОСТЬ

Авторы выражают благодарность всем сотрудникам, участвовавшим в создании препарата Бетарен 22, МКЭ, а так же всем, кто участвовал в постановке и проведении опытов с этим гербицидом: Силаеву А.И., Стаченкову Б.Г., Савве А.П., Веневцеву В.З. и другим. Мы также признательны руководству ГНУ ВИЗР и фирмы «Щелково Агрохим» за предоставленную возможность проведения этих исследований и их финансовое обеспечение.

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WILL CLIMATE CHANGE ALTER THE HERBICIDE USE

Katarina Jovanović-Radovanov[1], Gorica Vuković[2], Bojana Špirović[1] and Vojislava Bursić[3]

[1]University of Belgrade-Faculty of Agriculture-Zemun
[2]Institute of Public Health of Belgrade
[3]University of Novi Sad-Faculty of Agriculture
katarinajr@agrif.bg.ac.rs

ABSTRACT

The ongoing climate change is manifesting through domination of two seasons (winter and summer) with often very short and untypical spring and autumn. Such situation triggered our interest in both efficacy and safety of herbicide applications. Metribuzin is commonly used triazinone herbicide for early and very effective weed control in alfalfa.

The trials were conducted in 2013 on two locations: Boljevci and Zemun Polje. Two types of metribuzin formulations (WP and WG) were applied in two recommended doses (350 and 700 g a.i./ha), including the phytotoxicity treatment (1400 g a.i./ha as simulation for spray overlapping) and untreated check.

Efficacy and phytotoxicity assessments were performed twice during the spring time. Just before the harvest plant samples of alfalfa were collected for laboratory testing on residue level. Pesticide residues were determined by liquid chromatography coupled with tandem mass spectrometry.

The efficacy of both metribuzin formulations was at expected level. The prolonged cold weather induced retardation of metribuzin metabolism in alfalfa, resulting in significant level of phytotoxicity. At the time of the first evaluation growth reduction was 10 %, 15 % and over 30% for treatments of 350 g a.i./ha, 700 g a.i./ha and 1400 g a.i./ha, respectively. At the time of the second evaluation, there were no differences in alfalfa stand for recommended doses treatments and untreated check, but plants were still chlorotic and shorter (over 10 %) in treatment with 1400 g a.i./ha.

Chemical analysis showed that residue levels were in correlation with doses applied (in a range from 0.03 mg/kg to 1.18 mg/kg) and over MRL for treatments of 700 g a.i./ha and 1400 g a.i./ha.

Key words: metribuzin, efficacy, phytotoxicity, residue level

INTRODUCTION

Metribuzin [4-amino-6-tert-butyl-3-(methylthio)-as-triazin-5(4H)-one] is commonly used triazinone herbicide for early and very effective weed control in alfalfa (*Medicago sativa* L.). It is used to control small - seeded grasses and broadleaf weeds in a variety of crops.

In established stands of alfalfa grown for hay, pasture, dehydration, or seed, producers often prefer a residual herbicide such as metribuzin, as opposed to a short-lived post emergence herbicide, because a broader spectrum of weeds is controlled by a combination of immediate and residual herbicide action. Although established alfalfa could be damaged initially by spring application of metribuzin (Warnes et al, 1977; Waddington, 1980, Wilson, 1981) in the form of temporary stunting or minor visible injury, recovery usually is rapid (Malik et al., 1993).

Metribuzin is absorbed by both root and leaves, but moves throughout the plant only when absorbed by roots. Once in the plant, it interrupts carbohydrate synthesis by inhibiting photosynthesis (inhibition of electron transport in photo system II). A yellowing of the interveinal areas of the leaves is generally the first visual symptom to appear. The chlorosis progresses until entire leaves are affected and plant death occurs (in susceptible species) (Colquhoun, 2001). Reduced effectiveness for pre emergence weed control could result under high rainfall conditions in low organic soils like sandy loam due to leaching below the zone of weed seed germination (Sharom and Stephenson, 1976).

Selectivity of metribuzin is primarily based on metabolic degradation which proceeds through two phases. Phase I metabolic reactions include N-deamination, sulfoxidation and demethilation resulting in formation of nonpolar metabolites with reduced or modified phytotoxicity. Phase II reactions include N-glycosylation, acylation of N-glucoside conjugate with malonic acid, and conjugation of metribuzin sulfoxide with homoglutatione resulting in loss of herbicidal activity. Selectivity could be reduced in case of high precipitation especially on soils where water logging occurs.

The persistence of metribuzin in the soil is highly variable and dependent upon soil type and climatic conditions. Metribuzin is primarily degraded by microbial action and degradation in soil appears strongly influenced by microbial activity (Sharom and Stephenson, 1976) and by those soil factors that influence microbial activity, such as temperature, moisture and nutrient levels (Peter and Weber, 1985). The average field half-life is in the 30 to 60 days range (Hyzak and Zimdahl, 1974; Bowman, 1991; Gallaher and Mueller, 1996; Lechon et al., 1997; Di et al., 1998), but depending on conditions, it can last up to 75 days (Sharom and Stephenson, 1976; Bowman, 1991; Gillespie et al, 2011) and even up to 145 - 149 days (Webb and Aylmore, 2002; Henriksen et al., 2004). Allen and Walker (1987) concluded that metribuzin degradation rates were controlled by a combination of microbial activity, availability of herbicide in the soil solution and some component of the particle size distribution (sand, silt, or clay). Metribuzin persistence may be affected by volatility and/or photodecomposition losses under favourable field conditions, especially shortly after application. Method of application and conditions soon after application should be important considerations when attempting to predict or assess metribuzin carry-over problems (Jensen et al., 1989).

However, it has been shown that the degradation of metribuzin is highly temperature-dependent. Half-lives increase 6 to 11 times when lowering the temperature from 20 - 25°C to 5°C (Lechon et al., 1997; Bouchard et al, 1982; Sharratt and Knight, 2005). In laboratory studies, increased soil temperature and water content corresponded to increased degradation rates (Smith and Walker, 1989). Reduced rates of metribuzin dissipation could be the result of the effect of low temperature and/or soil water on microbial activity. Other factors reported to influence degradation of metribuzin, such as soil pH (Ladlie et al., 1976) and depth within the soil profile (Bouchard et al, 1982), also influence microbial activity. The magnitude of metribuzin sorption in surface soil is positively correlated to soil organic carbon content and soil clay content. Metribuzin sorption has also been shown to be inversely related to soil water content and soil pH. In addition, the amount of metribuzin adsorbed to soil has been found to increase with time. Desorption studies have demonstrated metribuzin sorption is not completely reversible (Sharon and Stephenson, 1976; Harper, 1988). This hysteresis has been reported to increase with soil organic matter content (Burgard et al, 1994) which indicates the presence of a slow long-term desorption and degradation.

It is a common observation that degradation rates below the root zone are significantly slower, primary due to lower microbial activity. Reported half-lives for metribuzin range from 50 to 222 d (Moorman and Harper, 1989; Di et al., 1998; Webb and Aylmore, 2002). It may be expected that the majority of metribuzin will dissipate during the growth season due to degradation, strong sorption, and bound residue formation.

The aim of this study was to examine influence of weather conditions on efficacy and selectivity of metribuzin applied in established alfalfa.

MATERIALS AND METHODS

The trials were conducted on two locations in 2013: Boljevci and Zemun Polje. In Boljevci, cultivar Banat was established in 2009, on chernozem type of soil. In Zemun Polje, cultivar Medijana was established in 2012, on chernozem type of soil. Two types of metribuzin formulations (WP and WG) were applied. Treatments included an untreated check and three metribuzin rates: 350 and 700 g a.i./ha (as label recommended application rates) and treatment with 1400 g a.i./ha (as simulation for spray overlapping) for each type of formulation. The trial treatments and evaluations were performed according the EPPO guidelines. Treatments were applied on 3rd and 4th of March. Application was made using a backpack sprayer delivering 240 l/ha at 2 bars with Tee jet XR 11002 flat fen nozzles. The plots were arranged in complete randomized block design with four replications. Individual plot was 5.5 m long by 4.5 m wide.

The beginning of vegetation period in 2013 was characterized with late occurrence of snow (on 27th and 28th of March – three weeks after herbicide application) and low temperatures for as long as the end of the first decade of April (table 1).

Table 1. Meteorological data

	Decade	Average temperature, °C			Total precipitation	
		min	max		rain, mm	snow, cm
Mart	I	5.1	14.2	9.7	4.8	-
	II	3.7	10.3	7.0	50.6	15
	III	2.4	8.8	5.6	42.7	7
April	I	4.6	11.5	8.1	17.0	-
	II	9.5	21.0	15.3	2.6	-
	III	14.8	27.8	21.3	2.2	-

Efficacy and phytotoxicity assessments were performed on March the 24th and 11th of April. Just before the harvest (May the 11th) plant samples of alfalfa were collected for laboratory testing on residue level.

Metribuzin was extracted from alfalfa hay using an extraction procedure based on the QuEChERS methodology (Anastassiades, 2003). Pesticides residues were determined by liquid chromatography coupled with tandem mass spectrometry. Analyses were perfomed using Agilent 1290 Infinity HPLC (High performance liquid chromatograph), with MS/MS Agilent 6460 Triple Quad detector. The column was a Agilent SB-C18, 2.1x50 mm, particle size 1.8 µm. The mobile phase was 0.1 % HCOOH in H_20 / 0.1 % HCOOH in MeOH (50:50).

RESULTS

On both locations there were 15 weed species present with more then 3 plants/m^2 what is considered to be the acceptable level of abundance for herbicide efficacy testing. At Boljevci there were 12 annual weed species (*Capsella bursa-pastoris*, *Lactuca serriola*, *Lactuca viminea*, *Lamium amplexicaule*, *Papaver rhoeas*, *Senecio vulgaris*, *Sinapis arvensis*, *Sonchus oleraceus*, *Stellaria media*, *Veronica arvensis*, *Veronica persica* and *Veronica polita*) and 2 perennials (*Convolvulus arvensis* and *Picris hieracioides*). At Zemun Polje there were also 12 annuals (*Ambrosia artemisiifolia*, *C. bursa-pastoris*, *Fumaria officinalis*, *Galium aparine*, *L. seriola*, *L. amplexicaule*, *P. rhoeas*, *S. vulgaris*, *S. arvensis*, *S media*, *V. hederifolia* and *V. persica*) and 2 perennials (*Cirsium arvense* and *Poa pratensis*). The efficacy of both metribuzin formulations was at expected level for all application rates tested (table 2 and 3).

Table 2. Efficacy of metribuzin formulations at Boljevci

Metribuzin application rates	I evaluation, 24.03.2013.				II evaluation, 11.04.2013.			
	350 g a.i./ha		700 g a.i./ha		350 g a.i./ha		700 g a.i./ha	
Plant species	WP	WG	WP	WG	WP	WG	WP	WG
	%							
CAPBP	100	91.7	100	100	93.8	100	100	100
CONAR	75.0	83.3	83.3	83.3	85.7	78.6	85.7	83.3
LACSE	91.6	100	100	100	100	92.8	100	100
LACVM	91.7	100	100	100	100	100	100	100
LAMAM	100	100	100	100	100	100	100	100
PAPRH	91.7	91.7	100	100	91.7	91.7	100	100
PICHI	75.0	66.7	75.0	75.0	69.2	69.2	76.9	76.9
SENVU	75.0	75.0	83.3	83.3	78.6	78.6	85.7	85.7
SINAR	100	100	100	100	100	100	100	100
SONAS	61.5	69.2	69.2	76.9	85.7	78.6	85.7	85.7
SONOL	76.9	76.9	84.6	84.6	89.5	78.9	89.5	84.2
STEME	100	93.8	100	100	99.9	100	100	100
VERAR	100	100	100	100	100	100	100	100
VERPE	100	100	100	100	95.0	95.0	100	100
VERPO	100	100	100	100	100	100	100	100

The plots were visually inspected 14 and 28 days after treatment and rated for foliar injury symptoms. No interveinal chlorosis was observed on the untreated check plots, but significant injury symptoms were present on metribuzin treated plots. The prolonged cold weather induced retardation of metribuzin metabolism in alfalfa, resulting in significant level of phytotoxicity. At the time of first evaluation both chlorosis and growth reduction were recorded for all treatments applied. At the time of second evaluation, there were no differences in alfalfa growth for recommended doses treatments, but plants were still chlorotic and shorter (over 10 %) in treatment with 1400 g a.i./ha (table 4).

An efficient, sensitive and specific method has been developed for the determination of metribuzin in alfalfa with LC-MS/MS. The calibration curve was determined using matrix–matched standards and exhibited excellent linearity from 50 – 5000 ng/mL. The linearity R^2 was over 0.999. The average recovery for 0.01 mg/kg was 84.3 ± 4.47 %, for 0.05 mg/kg was 91.5 ± 5.11 % and for 0.10 mg/kg was 93.2 ± 5.37 %. The validation parameters with the low LOQs of 0.01 mg/kg confirm that the method is suitable for the determination of pesticide residues in real alfalfa samples according to the regulations of the Serbian and EU MRLs.

Chemical analysis showed that residue level were in correlation with doses applied and over MRL for treatments of 700 and 1400 g a.i./ha (table 4).

Table 3. Efficacy of metribuzin formulations at Zemun Polje

Metribuzin application rates	I evaluation, 24.03.2013.				II evaluation, 11.04.2013.			
	350 g a.i./ha		700 g a.i./ha		350 g a.i./ha		700 g a.i./ha	
Plant species	WP	WG	WP	WG	WP	WG	WP	WG
	%							
AMBEL	100	100	100	100	95.2	90.5	95.2	95.2
CAPBP	100	91.7	100	100	100	100	100	100
CIRAR	53.8	38.5	61.5	46.1	58.8	29.4	64.7	47.1
FUMOF	100	100	100	100	100	100	100	100
GALAP	66.7	50.0	75.0	75.0	64.7	52.9	76.5	76.5
LACSE	83.3	100	91.7	100	92.8	100	100	100
LAMAM	100	100	100	100	100	100	100	100
PAPRH	91.7	91.7	100	100	92.9	92.9	100	100
POAPR	76.9	69.2	84.6	76.9	85.7	71.4	85.7	78.6
SENVU	78.6	85.7	85.7	85.7	78.6	85.7	85.7	85.7
SINAR	100	100	100	100	100	100	100	100
SONAS	83.3	75.0	83.3	83.3	84.6	76.9	84.6	84.6
STEME	100	91.7	100	100	100	94.4	100	100
VERHE	100	100	100	100	100	100	100	100
VERPE	100	100	100	100	95.0	95.0	100	100

Table 4. Metribuzin crop phytotoxicity and average residue level in alfalfa hay

Formulation type		WG			WP		
Phytotoxicity symptoms	Evaluation	Dose, g a.i./ha					
		350	700	1400	350	700	1400
Chlorosis, %	I	10.0	27.5	35.5	10.0	25.0	32.5
	II	0.0	0.0	20.5	0.0	0.0	20.0
	At harvest time	0.0	0.0	0.0	0.0	0.0	0.0
Growth reduction, %	I	10.5	15.5	35.0	10.0	15.0	33.0
	II	0.0	0.0	12.5	0.0	0.0	11.0
	At harvest time	0.0	0.0	0.0	0.0	0.0	0.0
Metribuzin content, mg/g	At harvest time	0.05	0.13	0.43	0.07	0.13	0.75

DISCUSSION

Prolonged cold weather after herbicide application induced significant visual injuries on alfalfa, but with no effect on crop growth and hay yield at the harvest time.

The MRL for metribuzin in alfalfa hay (fresh or dry) is 0.1 mg/kg. Higher recommended dose induced slightly higher residue level, but doubled dose as in case of overlapping induced significantly higher residue level. Herbicide application followed with prolonged low temperatures should be considered as a risk factor in exceeding MRLs posing the need in taking adequate monitoring and control measures.

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THE INFLUENCE OF TRIBENURON-METHYL, IMAZAMOX AND GLYPHOSATE ON BIOLOGICAL PRODUCTION OF *Ambrosia artemisiifolia* L.

Sava Vrbničanin*[1], Dragana Božić[1], Danijela Pavlović[2], Darko Stojićević[1], Katarina Jovanović-Radovanov[1], Marina Jevtić[1] and Katarina Stokić[1]

[1]University of Belgrade, Faculty of Agriculture, Belgrade, Serbia
[2]Institute for Plant Protection and Environment, Belgrade, Serbia
**sava@agrif.bg.ac.rs*

ABSTRACT

Ambrosia artemisiifolia L. (common ragweed) is an invasive weed species native to America and now widespread in central and southern Europe, with a tendency to spread eastward. In Serbia, it is most widespread in the Pannonian Plain, and along the valleys of large rivers and main roads to the south of Serbia. It is a weed of row crops (maize, soybean, sugar beet, sunflower, vegetables), rarely small grains, alfalfa, and clover fields and can very frequently be found in non-crop fields.

We examined the influence of herbicides including imazamox, tribenuron-methyl and glyphosate on biological production of *A. artemisiifolia*: plant height, dry weight and seed production under field conditions. Seedlings were produced in nursery, and when the seedlings were at the 2 true leaves growth stage, they were transplanted in the field. Humidity was provided daily by watering the experiment. The application of imazamox (Pulsar-40), tribenuron-methyl (SX-50 Express) and glyphosate (Glifol) in amounts of 1x, 0.5x, and 0.25x, was derived from the recommended rates when the plants reach the 4-6 leaf stage. Therefore, the herbicides were applied as follows: imazamox in rates 48, 24 and 12 g a.i. ha^{-1}, tribenuron-methyl in rates 22.5, 11.25 and 5.625 g a.i. ha^{-1} and glyphosate in rates 1440, 720 and 360 g a.i. ha^{-1}.

The effects of herbicides on plant height, dry weight and seed weight $plant^{-1}$ of ragweed differed depending on the type and amount of herbicides. The standard rates of tribenuron-methyl, imazamox and glyphosate significantly reduced the height (32%, 48%, 55%) and dry weight (70%, 79%, 94%) of ragweed, but lower rates also reduced the vegetative parameters, when compared to the untreated plants. Seed production of ragweed was also affected by the herbicides applied, but somewhat more poorly than vegetative parameters. The order of effectiveness of herbicides on *A. artemisiifolia* was: tribenuron-methyl < imazamox < glyphosate.

Keywords: common ragweed, efficacy, tribenuron-methyl, imazamox, glyphosate.

INTRODUCTION

Common or short ragweed (*Ambrosia artemisiifolia* L.) is an annual herb belonging to the Asteraceae family. It is a noxious invasive species native to America and now widespread in central and southern Europe, with a tendency to spread eastward (Smith et al., 2013; Nikolic et al., 2013). In Serbia, it is most widespread in the Pannonian Plain, along the valleys of large rivers and main roads to the south of Serbia (Vrbničanin et al., 2008). It is a weed of row crops (maize, soybean, sugar beet, sunflower, and vegetables), rarely small grain

crops, alfalfa, and clover fields and can be found very frequently in non-crop fields (Vrbničanin et al., 2008). Therefore, ragweed can cause serious yield and economic losses on arable fields. Chikoye et al. (1995) reported that ragweed causes yield losses of more than 50% in white bean, depending on the time of emergence and weed density. Unfortunately, in Serbia we have no exact data about crop yield losses caused by ragweed. In addition to crop yield losses and population expansion, the plant produces highly allergenic pollen that can cause serious health problems in both human and animal populations (Ognjenovic et al., 2013).

Generally, the aim of weed management is to keep the weed population at an acceptable level (below threshold), rather than to keep the crop totally free of weeds. Bearing in mind these principles, several studies have demonstrated satisfactory weed control and acceptable crop yields, when herbicides were used at lower than normally recommended rates (Zhang et al., 2000; Boström and Fogelfors, 2002; Barros et al., 2009). Reduced rates of herbicides are often sufficient to control weed density at or below the threshold levels. Below-labeled herbicide rates, in combination with some mechanical weed control measures, have proven to be an effective way to reduce the herbicide input in agricultural systems (Khaliq et al., 2011; Jovanović-Radovanov et al., 2013).

The objective of this study was to examine the influence of recommended and lower doses of imazamox, tribenuron-methyl and glyphosate on biological production (plant height, dry weight and seed weight plant^{-1}) of common ragweed under field conditions.

MATERIAL AND METHODS

Field experiment was conducted in 2013, near Belgrade at the Experimental Farm of the Faculty of Agriculture „Radmilovac" (44.75°N, 20.57°E) using seeds collected from mature common ragweed plants during the previous year. Seeds were cleaned and cold-stored until field planting. The experiments were conducted in a randomized complete block design with a split plot treatment arrangement in three replicates. Seedlings were produced in a nursery, and when the seedlings were at the growth stage of 2 true leaves, they were transplanted in the field by hand during the first week of May. Humidity was provided daily by watering the experiment. Plot size was 5x4 m. Interrow spacing was 25 cm and the distance between rows 70 cm. Precipitation and growing degree days (GDD, d°C = Σ [(T_{max} + T_{min}) / 2-Tbase]; Tbase = 10°C) are summarized in Table 1.

Table 1. Precipitation and GDD in 2013 at the experimental site.

Month	Precipitation (mm)	GDD
May	104.00	289.25
June	49.30	336.50
July	1624.00	436.65
August	1669.00	476.60
September	1967.00	232.00
Total	5413.30	1771.00

Ragweed was treated with herbicides at the growth stage of 4-6 true leaves, using a knapsack sprayer and 1004 flat fan nozzles to deliver a spray volume of 200 L water ha^{-1}. Application of imazamox (Pulsar-40, 40 g a.i. L^{-1}, SL, BASF, Germany), tribenuron-methyl (SX-50 Express, 500 g a.i. kg^{-1}, WG, Du Pont, Switzerland) and glyphosate (Glifol, Glifosat–IPA so -480 g a.i. L^{-1}, SL, Galenika fitofarmacija, Serbia) in amounts of 1x, 0.5x and 0.25x was derived from the recommended rates. Therefore, the herbicides were applied as follows: imazamox in rates 48, 24 and 12 g a.i. ha^{-1}, tribenuron-methyl in rates 22.5, 11.25 and 5.625 g a.i. ha^{-1} and glyphosate in rates 1440, 720 and 360 g a.i. ha^{-1}, and the control plants were not treated.

Visual damage estimates and measurements of plant height, dry weight and seed weight plant^{-1} (weight of achenes) were done on mature plants (at the end of September). Statistical analysis were carried out using STATISTICA 5.0 software. Data was subjected to one-way ANOVA (F-values) to evaluate the effects of the application of different herbicide rates on plant height, dry weight and seed weight plant^{-1} of ragweed, in comparison with the untreated control.

RESULTS AND DISSECTION

The effects of herbicides on plant height, dry weight and seed weight plant^{-1} of ragweed differed depending on the type of herbicide and application rate.

Tribenuron-methyl reduced the plant height by 32, 24 and 11%, and dry weight by 70, 66 and 38% in treatments with 1x (recommended), 0.5x (half of the recommended) and 0.25x (quarter of the recommended) herbicide application rates, respectively (Fig. 1a,b). The impact of tribenuron-methyl on seed weight plant^{-1} was lower in all treatments in relation to the vegetative parameters and ranged from 17.8% (0.25x) to 24.8% (1x) (Fig.1c). The applied rates of tribenuron-methyl reduced the plant height (F = 14.60059) and dry weight (F = 15.20742) significantly ($P < 0.01$), but not seed weight plan^{-1} (F = 0.26472) in comparison with the untreated control.

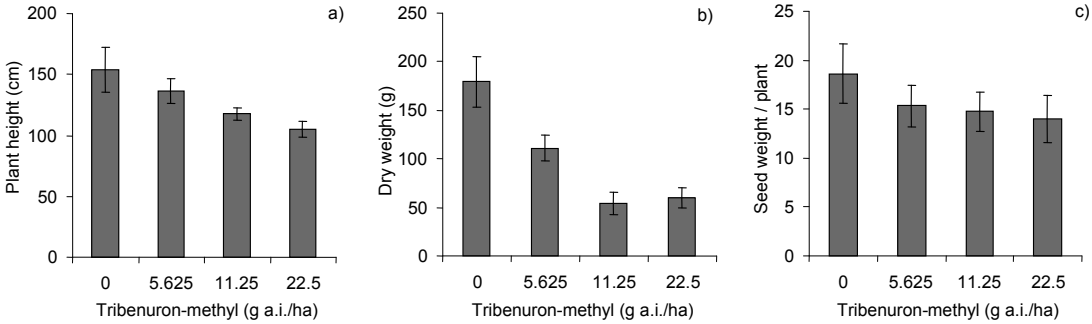

Figure 1. Effect of tribenuron-methyl on *A. artemisiifolia*: a) plant height (cm), b) dry weight (g), c) seed weight plant^{-1} (g)

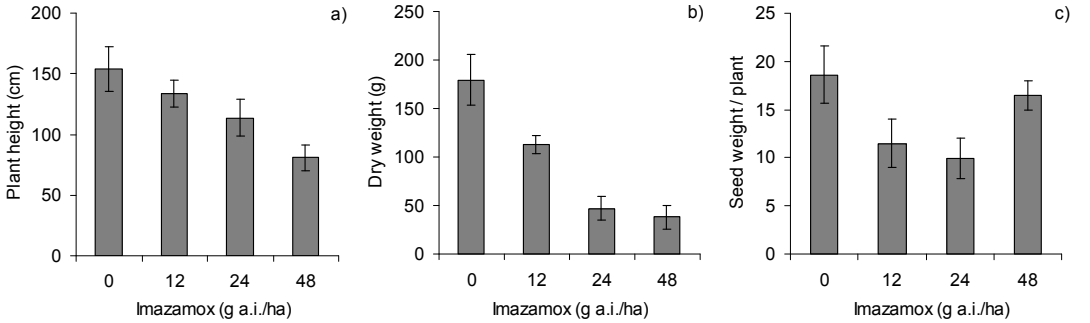

Figure 2. Effect of imazamox on *A. artemisiifolia*: a) plant height (cm), b) dry weight (g), c) seed weight plant^{-1} (g)

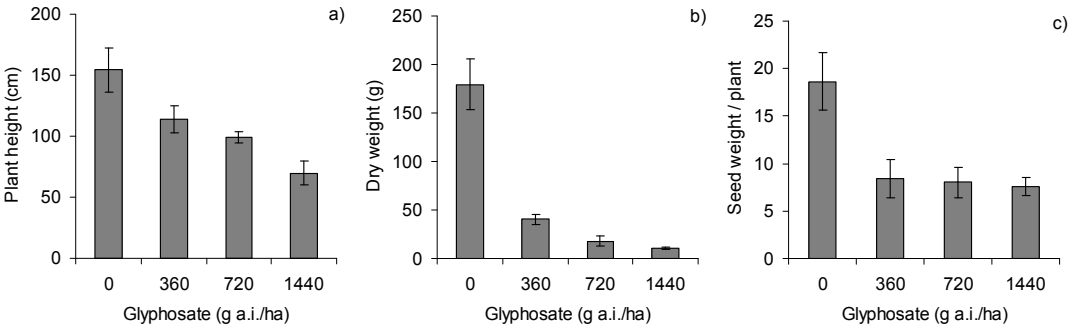

Figure 3. Effect of glyphosate on *A. artemisiifolia*: a) plant height (cm), b) dry weight (g), c) seed weight plant^{-1} (g)

Treatments with imazamox have reduced the dry weight, depending on the application rates by 79% (1x), 74% (0.5x) and 37% (0.25x), in regard to plant height by 48% (1x), 26% (0.5x) and 13% (0.25x), when compared with the untreated plants (Fig.2a,b). Therefore, imazamox has suppressed the height and weight of ragweed more strongly in comparison with the tribenuron-methyl. In addition, imazamox has also reduced the seed weight plant^{-1} of ragweed. However, that suppression was not in correlation with the increase of herbicide rates (12% (1x), 46% (0.5), 38% (0.25x)) (Fig.2c). The effect of all imazamox application rates on plant height ($F = 8.58544$) and dry weight ($F = 35.75991$) was statistically very significant ($P < 0.01$), while those differencies were insignificant for seed weight plant^{-1} ($F = 0.91515$), in comparison with the untreated control. The results are similar to the results of previous studies where Malidža et al. (2002) examined the effectiveness of imazamox in sunflower crop and found that this herbicide showed high efficacy in ragweed control, of 81-98%. Herbicide efficacy is largely dependent on the growth stage of ragweed and herbicide properties. Accordingly, some herbicides show good efficacy when applied in the initial stages of plant development, while in the later stages their efficiency significantly decreases (Chikoye et al., 1995).

Glyphosate reduced the plant height by 55% (1x), 36% (0.5x) and 26% (0.25x), and dry weight by 94% (1x), 90% (0.5x) and 77% (0.25x) in comparison with the untreated control (Fig.3a,b). When compared to previous treatments,

glyphosate suppressed the plant height and dry weight of ragweed more strongly than tribenuron-methyl and imazamox. Also, glyphosate reduced the seed weight plant^{-1} more efficiently than the other two herbicides and the level of this reduction ranged from 59% (1x) to 55% (0.25x) (Fig.3c). Furthermore, the effect of glyphosate on ragweed height (F = 14.56090) and dry weight (F = 63.74778) was significant ($P < 0.01$) in comparison to the untreated plants, while the differences in seed weight plant^{-1} between treatments with and without herbicide application were insignificant (F = 2.88109).

All three herbicides had a very significant impact on the reduction of ragweed vegetative parameters, while the effect on the seed weight plant^{-1} was somewhat lower. Accordingly, the order of effectiveness for the response of ragweed to the application of both recommended and lower rates of the herbicides was tribenuron-methyl < imazamox < glyphosate. The suppression of vegetative and generative weed production for a few years can lead to the incorporation of fewer weed seeds in the soil seed bank (Hartzler, 1996). Further research is needed to understand the long-term influence of the reduction of herbicide input, in combination with some mechanical weed control measures which have been proven to be an effective way in weed management in agricultural systems (Knežević et al., 2003; Khaliq et al., 2011).

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EFFECTS OF MIXTURES OF FUNGICIDE, INSECTICIDES, COMPLEX FERTILIZER AND ADJUVANT DEPENDING ON WATER HARDNESS

Slavica Vuković, Dušanka Inđić and Sonja Gvozdenac
University of Novi Sad, Faculty of Agriculture, Novi Sad vukovic@polj.uns.ac.rs

ABSTRACT

Components in the mixture have different physicochemical properties, purposes, modes of action and are often differently formulated. Currently, it has become a common practice to add non-pesticidal components in mixtures. However, there is little data on the effects of water quality on biological activity of plant protection products i.e. prepared spray liquids. The aim of this study was to determine the fungicidal effect, depending on the components in the mixture and water hardness. In this study, a mixtures of fungicide (azoxystrobin - Quadris 0.75 l/ha), insecticides (thiamethoxam - Actara 25-WG 0.07 kg/ha, cypermethrin - Cipkord 20 EC 0.3 l/ha), complex fertilizer (Mortonijc plus / M^+/ 3 kg/ha), and an adjuvant (Silwet L-77 0.1 l/ha) were used. Nine variants (eight mixtures and one single fungicide spray liquid) were tested. Spray liquids were prepared using water of different hardness: tap water from Novi Sad - slightly hard (15.4 d^oH) and well water from Adice - very hard (34.7 d^oH). *Alternaria solani* spore germination test on the glass was performed in four replications. The assessment was conducted by counting the number of non-geminated spores after 48 h and the results were expressed as the efficacy and processed with Two-way ANOVA. In the tap water, the efficacy of Quadris was 58.9%. The efficacy at the same level of significance was achieved with the following mixtures: Quadris + Cipkord 20-EC, Quadris + Actara 25-WG, Quadris + Silwet L-77, Quadris + Cipkord 20-EC + M^+ + Quadris + Actara 25-WG + M^+ and Quadris + Cipkord-20 EC + Silwet L-77. However, mixtures Quadris + Actara 25-WG + Silwet L-77 (77.7%) and Quadris + M^+ (76.2%) achieved significantly higher efficacy compared to the product Quadris alone. The efficacy of Quadris in well water was 73.5%. The efficacy at the same level of significance was achieved in all other variants which contained this fungicide (Quadris + Cipkord 20 EC, Quadris + Actara 25-WG, Quadris + Sillwet L-77, Quadris + Cipkord 20-EC + M^+, Quadris + Actara 25-WG + M^+, Quadris + Cipkord 20-EC + Sillwet L-77 + Quadris and Actara 25 + WG-Sillwet L-77), excluding Quadris + M^+. The efficacy of this spray liquid was 81.7% and was at significantly higher level than other mixtures containing Quadris, thus it may be concluded that only M^+ component synergized the effect of single fungicide compared to other variants. According to the results, the effect of Quadris on *A. solani* depended on the water quality and the mixture components.

Key words: mixtures, fungicide, insecticides, non-pesticidal components,water hardness

INTRODUCTION

Water hardness is a very important characteristic, and it is documented that it may negatively affect the efficacy of pesticides if it is harder, i.e contains more mineral substances (Snyder, 2010). The water hardness can also affect the compatibility of pesticide components in a mixture, as some are compatible in soft and incompatible in hard water (Igrc, 1983). Based on the experiments of Klokočar-Schmidt et al. (2002), preparation Folpan WP-50 alone and in combination with Pyrinex 48 EC or Sucip 20-EC expressed different inhibiting effect on germination of *A.*

alternata spores depending on water quality (well water-slightly alkaline, hard / Sremski Karlovci / and tap water-neutral, hard). Also, the inhibition was stronger and more uniform in the tap water, compared to well water. Given the above mentioned, the knowledge about water quality and its effect on pesticides is important for successful plant protection and rational agricultural production. Research regarding this problem is rare in Serbia and worldwide, thus the aim of this work was to determine fungicidal effect, depending on components in a mixture and water hardness.

MATERIAL AND METHOD

Water quality was analyzed in the Laboratory for Food and Feed Testing, Faculty of Technology, Novi Sad. Effects of mixtures of fungicide, insecticides, a complex fertilizer and an adjuvant, depending on the water quality, were tested in laboratories of Department for Plant and Environmental Protection, Faculty of Agriculture Novi Sad.

Water samples included in the tests – Two water samples were chosen: tap water (Novi Sad, GPS N 45° 14.833' E 19° 51.132') and well water (Adice, GPS N 45° 13.868' E 19° 46.870').

Water quality - Water quality (pH, hardness, conductivity, chloride, nitrite, nitrate, ammonium, calcium and iron) was determined according to standard methods (Table 1).

Pesticides, complex fertilizer, adjuvant and mixtures - fungicide and mixtures with insecticides, complex fertilizer (in the following text - M^+) and adjuvant, and applied rates are presented in Tab 2.

Test organism - fungus *Alternaria solani* (Sora uer) causes early blight of pepper, tomato, eggplant and potato. The isolate originated from tomato seed and was cultivated on PDA medium during the incubation for 7 days at 20 ± 2 °C with light regime light/dark 12/12 h. The colony was brown to black with a large number of conidia that were formed individually. Conidia were elongated, straight or slightly bent, with elliptical or oval body that gradually narrows towards the neck. The color varied from light olive-green to brown (Ivanovic, 1992).

Table 2. Fungicide, insecticides, complex fertilizer, adjuvant, their mixtures and application rates

Pesticides and non-pesticidal components	Application rates (kg, l/ha)/300 l water
Quadris	0.75 l/ha
Quadris+Cipkord 20-EC	0.75 l/ha+0.3 l/ha
Quadris+Actara 25-WG	0.75 l/ha+ 0.07 kg/ha
Quadris+ M^+	0.75 l/ha+3.0 kg/ha
Quadris+ Silwett L-77	0.75 l/ha+0.1 l/ha
Quadris+Cipkord 20-EC+M^+	0.75 l/ha+0.3 l/ha+ 3.0 kg/ha
Quadris+ Actara 25-WG+M^+	0.75 l/ha+0.07 kg/ha+ 3.0 kg/ha
Quadris+Cipkord 20-EC+ Silwett L-77	0.75 l/ha+0.3 l/ha+ 0.1 l/ha
Quadris+Actara 25 WG+ Silwett L-77	0.75 l/ha+0.07 kg/ha+ 0.1 l/ha

Fungicidal effect - The effect of fungicide and mixtures with insecticides, complex fertilizer and adjuvant in the mentioned waters was assessed using germination method on glass (Blumer and Kundert, 1951; Šovljanski and Klokočar-Schmidt, 1976, Stojšin, 1980). Tests were conducted in four replicates. In the control treatment water without fungicides was used. Assessment of germinated and non germinated spores was performed after 48 h. Fungicidal effect was expressed as the percentage of non germinated spores compared to the total number of examined spores.

Data processing and statistical analysis – Results on fungicidal activity were expressed in relative values (percentage of non germinated spores) and corrected according to Schneider Orelli (1947). The percent of non germinated spores was transformed in probit and arcsin $\sqrt{percent}$. The differences between the efficacy of spray liquids depending on the water quality and the components in mixtures were tested using analysis of variance, for confidence interval 95% (Statistica, 2008).

Table 1. The quality of water used in the experiments

The sampling sites (GPS)	Conc. of ions H, pH	hardness d° H	conductivity μ S/cm	chlorides (Cl) mg/l	NO_2^- mg/l	NO_3^- mg/l	NH_3 mg/l	Ca^{2+} mg/l	Fe^{2+3+} mg/l
Novi Sad (tap water)	7.42	15.4	641	26.0	0.002	2.8	0.01	78.4	0.02
Adice (well water)	7.55	**34.7**	1470	61.2	**2.5**	36.0	0.0	74.8	<0.05
II class *	***6.8-8.5***	**	***2500***	***200***	***0.03***	***50***	***0.1***	***200***	0.3

* - maximum allowable values for the water of II quality class (Anonimus, 1998);
** - scale of hardness (0-4 very soft; 4-8 slightly soft; 8-16 slightly hard; 16-30 hard; >30 very hard)

RESULTS

Test results on the efficacy of fungicide Quadris for *A. solani*, depending on the quality of water used to prepare the spray liquids are presented in Tables 3 and 4. Tables contain information on the significance of differences between efficacy of different preparations and mixtures, expressed as percent, probit and transformed into arcsin values. In the tap water / Novi Sad / (neutral / pH 7.4 / slightly hard /15.4 d° H /), the efficacy of preparation Quadris was 58.9% and of other combinations ranged from 45.8 to 77.7% (Table 3). The same level of efficacy with the preparation Quadris was achieved using mixtures: Quadris + Cipkord 20 EC, Quadris + Actara 25-WG, Quadris + Silwet L-77, Quadris + Cipkord 20-EC + M^+, Quadris + Actara 25-WG + M^+ and Quadris + Cipkord EC-20 + Silwet L-77. Preparation Quadris had significantly lower efficacy when used single compared to mixtures Quadris + M^+ (76.2%) and Quadris + Actara WG-25 + Silwet L-77 (77.7%). It is interesting to compare the efficacy of mixtures Quadris +Actara WG-25 (67.8%), Quadris + Silwet L-77 (57.3%) and Quadris + Actara WG-2 5+ Silwet L-77 (77.7%) with the preparation Quadris (58.9%), which leads to the conclusion, that the three component spray liquid achieved significantly higher efficacy compared to binary one. The efficacy of Quadris in the well water / Adice / (slightly alkaline / pH 7.55 / very hard to /34.7 d° H/, with a high content of nitrite /2.5 mg / l / 80 fold the MAC value for water of class II) was 73.5 %, and of all other variants ranged from 69.6 to 81.7% (Table 4). On the same level of significance with the efficacy of Quadris were: Quadris + Cipkord 20 EC, Quadris + Actara 25-WG, Quadris + Sillwet L-77, Quadris + Cipkord 20-EC + M^+, Quadris + Actara 25-WG + M^+, Quadris + Cipkord EC-20 + Sillwet L-77 and Quadris + Actara 25-WG + Sillwet L-77, with the exception of spray liqid Quadris + M^+. Spray liqid Quadris + M^+

Table 3. Efficacy (%) of Quadris and its mixtures in tap water (Novi Sad*) for *A. solani*

Products and mixtures	Concentration (%)	Average values		
		efficacy (%)	probit	arcsin $\sqrt{percent}$
Quadris	0.25	58.9 (±6.6) a**	5.28 (±0.25) a**	50.2 (±3.9) a**
Quadris+Cipkord 20-EC	0.25+0.1	65.6 (±8.6) a	5.42 (±0.24) a	54.2 (±5.2) a
Quadris+Actara 25-WG	0.25+0.023	67.8 (±7.4) a	5.48 (±0.21) a	55.5 (±4.6) a
Quadris+Mortonijc plus	0.25+1	76.2 (±5.4) b	5.73 (±0.16) a	60.9 (±3.6) b
Quadris+Silwet L-77	0.25+0.033	57.3 (±23.8) a	5.20 (±0.65) a	49.4 (±14.4) a
Quadris+Cipkord 20-EC+Mortonijc plus	0.25+0.1+1	69.1 (±8.7) a	5.51 (±0.24) a	56.4 (±5.4) a
Quadris+Actara 25-WG+ Mortonijc plus	0.25+0.023+1	59.4 (±3.1) a	5.25 (±0.08) a	53.4 (±6.4) a
Quadris+Cipkord 20-EC+ Silwet L-77	0.25+0.1+0.033	45.8 (±12.2) a	4.90 (±0.30) a	42.6 (±7.1) a
Quadris+Actara 25-WG+ Silwet L-77	0.25+0.023+0.033	77.7 (±7.5) b	5.78 (±0.26) b	62.1 (±5.4) b
LSD 0.05		16.3	0.46	10.5

* neutral (pH 7.4); slightly hard (12.9 d° H)
**efficacy was tested in comparison to efficacy of Quadirs used as a single preparation

Table 4. Fungicidal efficacy (%) of Quadris and their mixtures in well water (Adice*) for *A. solani*

Products and mixtures	Concentracion (%)	Average values		
		efficacy (%)	probit	arcsin $\sqrt{percent}$
Quadris	0.25	73.5 (±4.1) a**	5.64 (±0.13) a**	59.1 (±2.6) a**
Quadris+Cipkord 20-EC	0.25+0.1	76.3 (±6.9) a	5.73 (±0.22) a	61.1 (±4.6) a
Quadris+Actara 25-WG	0.25+0.023	74.2 (±5.2) a	5.66 (±0.16) a	59.6 (±3.4) a
Quadris+Mortonijc plus	0.25+1	81.7 (±5.1) b	5.92 (±0.21) b	64.9 (±3.9) b
Quadris+Silwet L-77	0.25+0.033	77.0 (±5.7) a	5.76 (±0.19) a	61.5 (±4.0) a
Quadris+Cipkord 20-EC+Mortonijc plus	0.25+0.1+1	78.5 (±5.0) a	5.80 (±0.16) a	62.4 (±3.5) a
Quadris+Actara 25-WG+ Mortonijc plus	0.25+0.023+1	69.6 (±3.6) a	5.52 (±0.1) a	56.6 (±2.3) a
Quadris+Cipkord 20-EC+ Silwet L-77	0.25+0.1+0.033	76.4 (±2.7) a	5.72 (±0.09) a	60.9 (±1.8) a
Quadris+Actara 25-WG+ Silwet L-77	0.25+0.023+0.033	77.6 (±3.3) a	5.76 (±0.17) a	61.8 (±2.2) a
LSD 0.05		6.37	0.21	4.36

* slightly alkaline (pH 7.55); very hard (34.7 d°H) - nitrite content is increased 80-fold (2.5 mg / l)
**efficacy was tested in comparison to efficacy of Quadirs used as a single preparation

achieved efficacy (81.7%) at a significantly higher level than the other mixtures or Quadris alone. Thus may be concluded that only M^+ synergized the effect of Quardis compared to the other variants. The abovementioned conclusion is confirmed by the comparative analysis of efficacy, expressed in %, probit and arcsin values. In the well / Adice / and tap water / Novi Sad / complex fertilizer M^+ significantly increased the efficacy of the fungicide Quadris, however, in the tap water with the addition of adjuvant (Quadris + Actara 25-WG + Silwet L-77) the efficacy was significantly increased compared to single fungicide, which is likely due to differences in the quality of tested water samples. Based on the analysis of two-factorial experiment (fungicide and water) the efficacy of fungicide Quadris and the average efficacy in the tested waters, regardless on the components in spray liquids but depending on the water quality are presented in Table 5. According to the average values independently of the components in spray liquids, the efficacy of Quadris was at significantly lower level in the tap water compared to the efficacy in well water.

Tabela 5. Efficacy of Quadris and its mixtures for *A. solani* depending on water quality

Water	Efficacy (%) of Quadris	Average efficacy (%) of all variants which contain Quadris
Novi Sad (tap water)	58.9 a	64.2 a
Adice (well water)	73.5 b	76.1 b
LSD 0.05%	8.07	3.51

DISCUSSION

Preparation Quadris, based on azoxystrobin, was chosen for this study because it is stable in acidic and neutral media, while is unstable at higher pH values (Roberts and Hutson, 1999; Park and Chong, 2009). Also, prior to fungicide selection the spectrum of biological activity of this preparation was considered. The efficacy of Quadris for *A. solani* in slightly alkaline, very hard well water (Adice) was 73.5% and in neutral, slightly hard tap water (Novi Sad) was 58.9%. The efficacy of preparation Quadris in tap water (Novi Sad) was at a significantly lower level compared to the efficacy in the tap water (Adice). The reason might be the quality of water or the presence of chlorine in it. According to Coping (2009) it is not recommended to use the water that contains chlorine in the application of biological products including those based on fungy. At the same level of significance with the efficacy of Quadris were all other mixtures, with the exception of spray liquids Quadris + M^+ in well water, Quadris + M^+ and Quadris + Actara 25-WG + Silwet L-77 in the tap water, which achieved efficacy at a significantly higher level than Quadris alone. Based on these findings it can be concluded that the M^+, Actara 25-WG and Silwet L-77 synergized the effect of the preparation Quardis, i.e. caused increased action of fungicide in mixtures (Brindley and Selim, 1984), which is consistent with studies of El-Saidy et al., (1986), Indic et al. (1994), Vukovic (2011), who stated that the improvement of fungicidal effect of some pesticides can be achieved by adding non-pesticidal components (additives, adjuvants, vegetable and mineral oils, herbal extracts, etc.) in a tank-mix. Bearing in mind that Quadris has systemic action and that there is a lot of justification for the application of tested mixtures, it is necessary to study further both physicochemical and biological properties of mixtures for determining the one with the highest efficacy in the water of a certain quality and optimum components content, within strategies for delaying the resistance towards pests.

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THE EFFECTS OF THUJA AND FIR ESSENTIAL OILS ON HOUSE MOUSE FOOD INTAKE

Goran Jokić[1], Rada Đurović-Pejčev[1], Tanja Šćepović[1,2], Marina Vukša[1], Suzana Đedović[1] and Bojan Stojnić[3]

[1] Institute of Pesticides and Environmental Protection, 11080 Zemun, Banatska 31b, Serbia
E-mail: jokicg@ptt.rs
[2] Grant-holder of the Ministry of Education, Science and Technological Development of RS
[3] University of Belgrade, Faculty of Agriculture, Serbia

SUMMARY

The house mouse, a worldwide commensal rodent pest, can produce high damage on properties, as well as to crops during vegetation and to agricultural commodities after harvest. By now, the influence of some derivates isolated from coniferous trees on small rodent species diets has been confirmed. In this study we tried to determine the effects of thuja (Fam. Cupressaceae) and fir (Fam. Pinaceae) essential oils as natural sources of strong-smelling organic compounds on house mice diet.

Essential oils of the tested plants were isolated by hydrodistillation using a Clevenger-type device. A gas chromatographic-mass spectrometry (GC-MS) analysis of the obtained oils showed a predominant presence of terpenoid compounds, especially alpha-pinene (in thuja oil) and p-mentha-2,4(8) diene (in fir oil).

Under standard laboratory conditions, the effects of essential oils of thuja (isolated from needles and seeds, separately) and fir (isolated from needles) on house mice diet were examined. The tested baits were prepared following an EPPO/OEPP standard method (2004) by mixing placebo and 1 ml of essential oil previously diluted in 20 ml of alcohol. Over a period of four days, wild-born house mice chose between placebo and tested bait. Water was available *ad lib.*

The fir essential oil had a repellent effect in house mice diet (bait acceptance and palatability were 24.07 and 0.3170, respectively), while thuja oil (both plant organs) showed neutral or slightly attractive activity (maximum bait acceptance and palatability were 35.15 and 0.5421, respectively). Considering the composition of essential oils, we concluded that p-mentha-2,4(8) diene had a repellent effect in house mice diet. However, the conclusion requires a check in additional trials.

Keyword: essential oil; thuja; fir; house mouse

INTRODUCTION

Mus musculus domesticus, the house mouse, is a worldwide commensal rodent pest that can produce high damage on properties, as well as to crops during vegetation and agricultural commodities after harvest (Pimentel, 1991; Singleton et al., 2010).

Various non-chemical and chemical rodent pest management strategies are currently being used to reduce damage caused by commensal rodent pests. Generally, chemical methods are most common used against commensal rodent pest (Buckle, 1994; Shirley et al., 1997). Some compounds can be used as additives, either repellents or attractants. Potentials of such compounds as bioactive chemicals may be assessed based on their acceptability and palatability to different target pest species.

The influence of some derivates isolated from coniferous trees on small rodent species diet has already

been confirmed (Ahn et al., 1995; Kelsey et al., 2009). Also, some derivates have been confirmed as having antimicrobial (Hong et al., 2004; Kim et al., 2013; Ervigit et al., 2014), insecticidal (Szolyga et al., 2014) or herbicidal activity (Amri, 2013). Some of them are used in medicine (Biswas et al., 2011).

The aim of this study was to investigate the possibility of application of thuja and fir essential oils as repellents or attractants in rodent baits. Our basic intention was to assess the acceptability and palatability of baits containing 0.1 % of essential oils to house mice in the laboratory.

MATERIAL AND METHODS

Animals

Wild-born individually caged house mouse adults were used in each trial. Over a period of three weeks, animals were acclimatized on food and water provided *ad lib*.

The animals were on standard diet for laboratory mice before and after trials. The trials were conducted under standard laboratory conditions (temperature 20-24°C, humidity 40-70%, and 14/10 h light/dark cycle). The oil baits were evaluated in a choice feeding test.

Placebo baits were prepared according to EPPO/OEPP (2004) methodology by mixing coarsely-cut cereal (wheat, barley and maize) and medium-ground oatmeal and adding 25 ml of pure alcohol. Alcohol was used for dissolving the essencial oils before mixing them with placebo bait. We tested the effects of placebo baits containing 0.1 % essential oils of thuja (Fam. Cupressaceae), isolated from needles and seeds separately, and fir (Fam. Pinaceae), isolated from needles.

Each trial lasted four days. The tested oils and placebo baits were offered in bowls at the opposite sides of cage. An amount of 10 g of each bait was offered daily. Bait consumption was measured daily. A new bowl was placed at the opposite site of each cage after measurement.

Essencial oils

Essential oils were isolated by hydrodistillation using a Clevenger-type device. A gas chromatographic-mass spectrometry (GC-MS) analysis of the obtained oils showed a predominant presence of terpenoid compounds, especially alpha-pinene and p-mentha-2,4(8) diene (Table 1).

Table 1. Main components (content higher than 10%) in the essential oils of tested plants

Fir, needles		
Compounds	RI*	Content (%)*
tricyclene	926	11,61
camphene	952	10,58
p-mentha-2,4(8)-diene	1086	**15,99**
terpinene-4-ol	1174	12,5
Thuja, needles		
α-pinene	941	**41,75**
δ-3-carene	1010	26,4
Thuja, seeds		
α-pinene	941	**43,75**
δ-3-carene	1010	34,83

*RIs (Retention Indexes) and contents (%) were obtained by GC-FID on apolar HP-5 column, while identification of each component was performed by GC-MS using the Wiley 7 library

Computation and statistical analyses

The effects of essential oils on bait acceptance and palatability to house mice were determined in a choice feeding test according to a formula by Johnson and Prescot (1994):

$$\text{Bait acceptance (\%)} = \frac{\text{total weight (g) of rodenticide bait eaten (T)}}{\text{total weight (g) of control bait (C) and rodenticide bait (T) eaten}} \times 100$$

$$\text{Palatability ratio} = \frac{\text{total weight (g) of rodenticide bait eaten (T)}}{\text{total weight (g) of control bait eaten (C)}}$$

A one-way analysis of variance and the Tukey-Kramer test for post hoc analysis were applied to analyse the influence of gender on daily bait consumption. Comparison was made using Statistica for Windows 6.0 (Stat Soft Italia, 1997) software package.

RESULTS

The most dominant of the identified compounds in fir oil were tricyclene (11.61%), camphene (10.58%) and p-mentha-2,4(8)-diene (15.99%) as monoterpene hydrocarbons, and terpinene-4-ol (12.50%) as oxygenated monoterpene. Monoterpene hydrocarbons (α-pinene and δ-3-carene) were dominant components in thuja needles and thuja seeds oils (Table 1).

Table 2. Acceptance and palatability of placebo bait containing 1 ml essential oil per 1 kg^{-1} to house mice in a choice feeding test

Gender	Thuja (needles)		Thuja (seeds)		Fir (needles)	
	Plain bait	Oil bait	Plain bait	Oil bait	Plain bait	Oil bait
Male	11.00±0.7*	5.55±0.6	11.03±0.5	6.02±0.5	11.47±0.6	2.34±0.5
Female	10.62±0.4	5.40±0.6	10.78±0.9	5.53±0.9	7.27±0.7	3.60±0.7
♂ between groups	A	a	A	a	A	b
♀ between groups	A	a	A	a	B	a
Bait acceptance (%)	33.57		34.83		24.07	
Palatability	0.505		0.534		0.317	

* Average mean and standard error of daily bait consumption

The lowest average daily intake of 3.45 g/day was found in animals taking fir oil bait, while the highest intake was recorded in mice taking thuja seeds oil bait, 4.26 g/day. Daily consumption of fir oil bait by male house mice was statisticaly different ($F_{2,27}$=13.28; p<0.05) from daily consumption of thuja oils (both origin) baits. Regarding female house mice, their daily consumption of plain baits was statisticaly different ($F_{2,15}$=13.28; p<0.05) from daily consumption of thuja oils (both origin) baits.

DISCUSSION

Data from our analysis of contents of essential oils were consistent with available literature sources (Loizzo, et al., 2008; Tsiri, et al., 2009; Tognolini, et al., 2006).

The average daily bait intake in all experimental groups of mice (3.5-4.3 g/day) was nominally lower than the estimated daily requirement (5.7 g/day) of the house mouse (European Commision, 2002), but it caused no change of animal behaviour or any lethal effect in any of the experimental groups of animals. Even though alpha pinene is known to have caused changes in rat behaviour by raising their body temperature under induced stress (Akutsu et al., 2003) such change did not occur in our tests with thuja oils (both origins).

Essential oils from different coniferous plants which contain volatile terpenes have antifeedant or repelent properties (Langenheim, 1994). In our research, the fir essential oil had a repellent effect on house mice diet (bait acceptance and palatability were 24.07 and 0.317, respectively), while thuja oil (both origins) showed neutral or slightly attractive activity (maximum bait acceptance and palatability were 35.15 and 0.5421, respectively) (Table 2). Under the same laboratory conditions, baits with 1% of lavender, fencel, scots pine and thyme essential oils had shown similar repelent effects on laboratory Swiss mice (Jokić et al., 2013). Many other studies have confirmed repellent or deterrent effects of oils with similar chemical composition (i.e. terpenes, monoterpenes) on different animal species (Yun et al., 1998; Shirley et al., 1997; Bell and Harestad 1987). Discussing data from their no-choice feeding test, Shirley et al., (1997) pointed at a possibility of animals getting used to oils in their diet. In our choice tests, the animals preferred food without oils.

Further research should focus on examining the effects of fir oil on daily consumption by house mice in no-choice feeding tests. Considering their potentially high volatility, the period of oil persistence should also be determined.

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INTEGRATED PROTECTION OF FRUIT CROPS

ИНТЕГРИРОВАННАЯ ЗАЩИТА ФРУКТОВЫХ НАСАЖДЕНИЙ

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OPTIPAON, A DECISION SUPPORT SYSTEM TO PREDICT THE RISK OF PEACOCK EYE OF OLIVE IN SOUTHERN FRANCE

C. Roubal[a], S. Regis[a] and P.C. Nicot[b]

[a]ONPV. Quartier Cantarel, BP 70095, F-84143 Montfavet CEDEX. France
[b]INRA, UR407 Pathologie végétale, F-84140 Montfavet, France. philippe.nicot@avignon.inra.fr

ABSTRACT

Peacock eye, caused by *Fusicladium oleagineum*, is a major disease in most olive production regions, including southern France. Its control relies on up to 6 treatments per season. A more accurate evaluation of disease risk would allow to reduce the frequency of treatments.

Work was conducted to develop a field-operational model for disease prediction based on climatic conditions, using data form a 10-year survey. As disease outbreaks are known to be linked to rain, models were evaluated for their ability to predict if infection would occur following a rain event, depending on air temperature and duration of relative humidity above 85%. We examined a total of 134 rain events followed by confirmed leaf infection and 191 rain events not followed by detectable infection. The field data were adequately fitted with two regression models describing high boundary values of high humidity duration, above which no infection occurred over the temperature range, and low boundary values below which no infection occurred. One problem associated with risk prediction of peacock eye is the long latent period (time between infection and the first detection of leaf spots) of this disease. We thus developed a second model to relate the duration of the latent period as a function of air temperature after the beginning of rain. Used together, these two models allowed to predict the numbers of ongoing latent infections. They were included in a decision support system (DSS), referred to as „OPTIPAON", to help farmers optimize the number and timing of their treatments. In addition to estimating the ongoing latent infections, this DSS takes into account six other risk factors related to the location of the orchard and its recent history. This system is currently being evaluated by a group of farmers in Provence.

Keywords: Expert system, *Spilocaea oleagina*, IPM, infection, incubation

INTRODUCTION

Olive production covered over 10 million ha worldwide in 2013 (FAOSTAT – freely accessible at http://faostat.fao.org/). Peacock eye, caused by *Fusicladium oleagineum*, is a major disease in most olive production regions, including France, where production is located in the South and covers 40,000 ha. In this region, the fungus essentially causes leaf spots and may result in substantial defoliation in severely attacked orchards. The fungus does not produce sexual spores but numerous cycles of conidial production can occur year round. Disease results from leaf infection by airborne conidia and is dependent on the concomitant occurrence of rain and mild temperatures (Miller, 1949). The risk periods in southern France are thus mostly restricted to spring and autumn. Disease control relies mostly on fungicides, requiring up to 6 sprays per season. One possibility to reduce the use of fungicides (usually copper) would be to limit treatments according to the actual risk of disease development. Relationships between climate parameters (temperature and duration of leaf wetness) and leaf infection or symptom development have been reported in previous studies (Obanor et al. 2008; Viruega et al., 2002, 2011). However, these data may be difficult to use directly for field prediction purposes, as they have been produced in stable, controlled conditions. Field conditions

can fluctuate widely and are usually heterogeneous within an orchard. Furthermore the reliability of wetness sensors for field use is often considered as questionable (Magarey et al, 2006; Sentelhas et al., 2005).

The purpose of the present study was thus to develop field-based disease prediction models, based on easily measured climatic conditions, and to integrate them into an expert system.

MATERIALS AND METHODS

Over a 10-year period, climatic parameters and the incidence of peacock eye were monitored weekly in an untreated orchard of southern France (Mas-de-la-Dame, in the Baux-de-Provence Valley). Air temperature, relative humidity (RH) and rain intensity (mm per hour) were recorded continuously with the help of climate sensors linked to a weather station. Disease incidence was assessed one to three times a week on samples of 100 leaves randomly collected in the orchard. The number of leaves with visible spots was recorded and leaves without symptoms were processed as described before to reveal latent infections (Roubal et al. 2013). These data were used to develop two types of models.

An initial step prior to model construction was dedicated to linking rain events and leaf infection events. Rain is known to be necessary for leaf infection, but infection will occur only if conditions during and after the rain are favourable. Each rain event between 1999 and 2009 was thus examined to determine if it resulted in an increase in the percentage of infected leaves in the orchard. This assessment was carried out according to two iterative steps described elsewhere (Roubal et al. 2013). These steps were based on known effects of temperature on (i) leaf infection by *F.oleagineum* and (ii) the duration of symptom development. This allowed to identify „high boundary values", representing data points with the longest duration of high humidity (RH>85%) below which infection never occurred after a rain. Similarly, „low boundary values" were identified, defining the shortest duration of high humidity above which infection always occurred after a rain (Figure 1).

The first modelling step consisted in performing regression analysis on the boundary values to establish the relationship between the occurrence of infection and two key climate parameters: (i) the average temperature during the rain and (ii) the duration of high humidity after the beginning of the rain. Six non-linear models were assessed and compared based on the standard error of their estimates and the correlation coefficients.

The second modelling step consisted in estimating the duration of the incubation period (the time needed for symptoms to appear after infection has occurred) as a function of air temperature after the beginning of an „infectious" rain. To this end, polynomial regression analysis was performed.

The two predictive models were then combined to build a software allowing the characterization of the successive cycles of disease and the prediction of symptom outbreaks based on observed and forecasted weather. However, actual risk could vary widely depending on other factors related to the specific location of the orchard and its recent history. For example, the microclimate at orchard level could be somewhat different from that at the reference weather station. To take these additional factors into account, a decision support system (DSS) was developed. The additional risk factors include the susceptibility of the cultivar, the mode of irrigation, the number of previously applied copper treatments, a global climatic risk linked to average rainfall in the production region, the type relief of the orchard and an estimation of disease incidence. This latter information must be provided online by the grower at every use of the DSS. Three risk levels are considered for each criterion (Table 1).

Table 1. Criteria used in the OPTIPAON decision support system to modulate risk assessment based on the specific conditions of an olive orchard.

Input criteria	Choice 1	Choice 2	Choice 3
Current disease incidence (% infected leaves)	< 10 %	>10 % &<20 %	> 20 %
Cultivar susceptibility	Low (Picholine)	Medium (Aglandau, Verdale)	High (Grossane, Tanche, Lucque, Bouteillan)
Water management	no irrigation	occasional irrigation	regular irrigation, humid soil
Climatic risk zone	Low risk (few disease cycles per year)	Medium risk	High risk (many disease cycles per year)
Environmental situation of the orchard	Open space, well ventilated	Flat land, few wind breaks	Confined, lowland, river, many windbreaks
Last year's treatments	None	1	2 or more

RESULTS

Among 376 rain events that occurred in the orchard between 1999 and 2009, 134 were identified as leading to confirmed leaf infection and 191 were clearly not followed by detectable infection by *F. oleagineum* (Figure 1). In addition, 51 rain events could not be clearly assigned and were not used for model construction.

The best models describing the low and high boundary values were the Logistic and the Vapor Pressure models, respectively. The equations and parameters of these models are presented in Table 2.

The best regression line for the prediction of the incubation period was obtained with a four-degree function. Its equation was:

$$D = 364.76 - 89.57 * T + 9.12 * T^2 - 0.43 * T^3 + 0.0078 * T^4$$

Where D and T represent the duration of incubation and the average daily temperature during incubation, respectively.

The software combining the two predictive models allows the user to assess the risk of disease development following a rain event. An example of output from this software for the whole 2013 growing season is shown on Fig. 2. Based on the temperature and RH during the day after rain onset, the first model provides an answer to the question „Is this rain going to lead to leaf infection"? If the model estimates that infection will occur, a red circle is shown on the graph, and a yellow

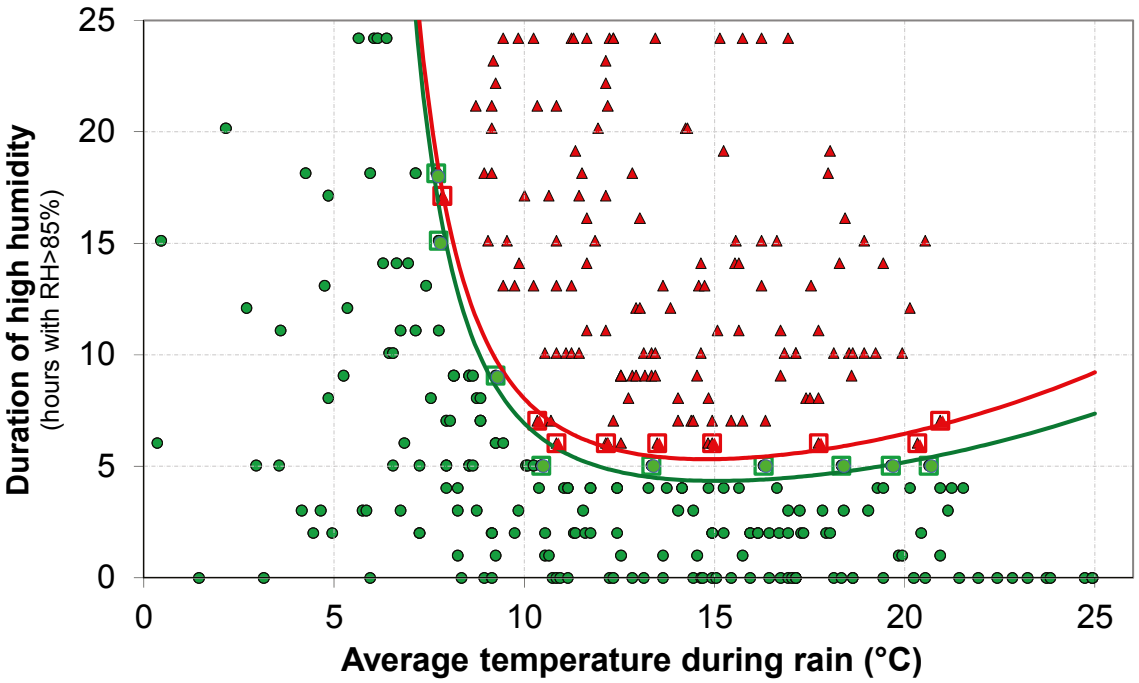

Figure 1. Characterization of rain events according to temperature and duration of high relative humidity after rain onset. Green circles (●) indicate rains that did not lead to infection while red triangles (▲) indicate those that did not lead to leaf infection. Green and red squares represent the „high" and „low boundary values" described in the Materials and Methods. The curves represent the regression lines.

Table 2. Regression models fitted to the boundary values described in Figure 1

Model	Equation	Parameters	Standard error	Correlationcoefficient
Vapor Pressure	y = exp(a+b/x+c*ln(x))	a = -16.4927 b = 72.8655 c = 4.8386	0.960	0.986
Logistic	y = a / (1 + exp (b – c*x))	a = 6.1582 b = -1069.84 c = 0.9398	0.418	0.996

horizontal bar indicates the incubation period, when symptoms remain invisible. Based on the average daily temperature after rain onset, the second model provides an answer to the question: „When will the symptoms become visible?" The estimated date is shown on the graph as a red triangle, which indicates the end of the incubation period.Overall, the user can easily visualize the different ongoing incubation periods and forecast periods when substantial outbreak of leaf spots will occur. Examples of such dangerous periods are shown as wide red bars on Fig. 2. Based on this knowledge, an Alert Bulletin is sent to growers and posted online to indicate the occurrence of a general risk.

Farmers can adapt the risk assessment to the specific situations of their own orchards by logging on OPTIPAON and entering the information needed for each of the 6 additional risk factors. The output of the DSS provides an orchard-specific risk index on a scale from 0 to 5. This information can then be used by the farmer to make a rational decision on the pertinence of spraying the orchard.

DISCUSSION

The analysis of weather and disease incidence data over a 10-year period has allowed the development of two complementary field-based biological models describing the relationship between easy-to-measure climate parameters and the infection of olive leaves by *F.oleagineum* and the appearance of symptoms in the orchard. These two predictive models have been validated with additional observations since 2010. They also have been used as a basis to evaluate the risk of disease outbreak, using data provided by more than 20 weather stations distributed throughout the olive production region, and to produceAlert Bulletins widely disseminated to French olive growers.

The development of the OPTIPAON DSS has provided each farmer the ability to adapt the risk assessment to the specific condition of their own orchards. Several elements constitute a favourable context for the wide adoption of OPTIPAON by farmers to devise rational strategies for the protection of their olive orchard and thus reduce the use of fungicides. The epidemic development of the disease is overall slow in southern France and practical damage thresholds are relatively high, with no significant leaf drop if the incidence of diseased leaves remains below 20% and an absence of yield loss if it remains below 10%. As a consequence, a possible occasional underestimation of disease risk by OPTIPAON could efficiently be corrected by the farmer at the occasion of the next Alert Bulletin.

This system is hosted on the website of CIRAME (Centre d'Information Régional Agrométéorologique) and is currently being tested by a group of farmers. Further improvements in the parameterization of the

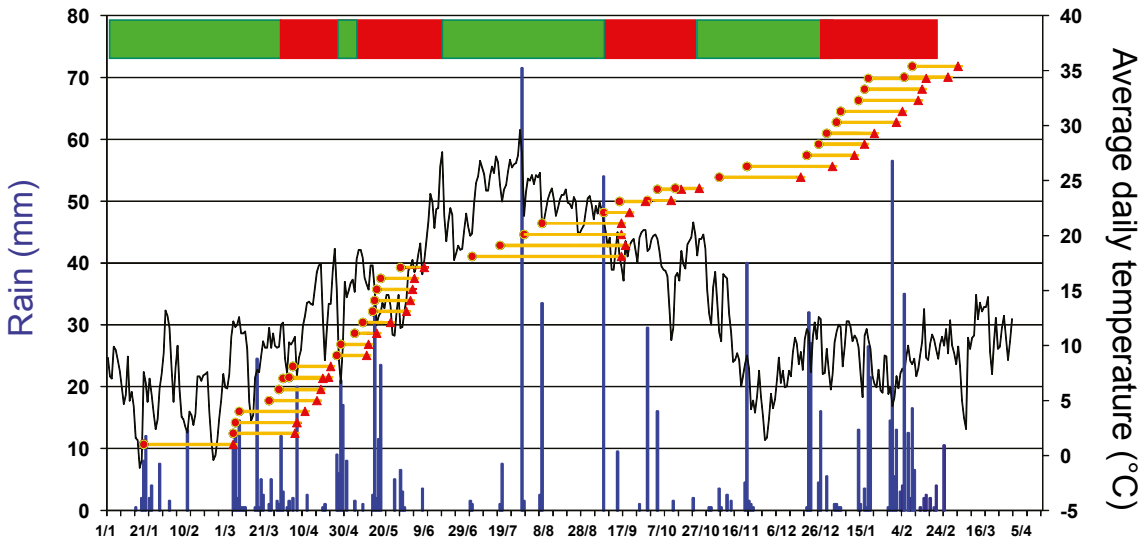

Figure 2. Example of output from the software developed to characterize the successive cycles of disease based on weather data for 2013. Red circles show the estimated dates of leaf infection. Yellow bars show the duration of the incubation period and red triangles the estimated dates of symptom appearance. Blue bars indicate daily rain amounts and the black line shows the average daily temperature. The wide bar above the graphs show the periods of estimated low (green) or high (red) risk based on the occurrence of ongoing disease cycles.

system are envisioned, using the data collected by the testers. In addition, the susceptibility level of a larger number of olive cultivars may be taken into account in the future, on the basis of an ongoing survey.

ACKNOWLEDGEMENTS

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EVALUATION OF TRUNK-INJECTED BACTERICIDES AND PROHEXADIONE-CALCIUM FOR ENVIRONMENTALLY FRIENDLY CONTROL OF FIRE BLIGHT (*Erwinia amylovora*) IN APPLES

Srđan G. Aćimović[1,*], Gayle C. McGhee[1], George W. Sundin[1] and John C. Wise[2]

[1]Michigan State University, Department of Plant, Soil and Microbial Sciences, 103 Center for Integrated Plant Systems, 578 Wilson Road, East Lansing, MI 48824-1311, USA
[2]Michigan State University, Department of Entomology, 206 Center for Integrated Plant Systems, 578 Wilson Road, East Lansing, MI 48824-1311, USA
**Corresponding author's e-mail: acimovic@msu.edu*

ABSTRACT

Trunk injection is a novel delivery method for plant protective compounds in agriculture. It could increase sustainability of fruit production through target-precise disease control. We evaluated trunk-injected antibiotics, copper chelate (CC), and prohexadione-calcium (PC) in control of fire blight on apples. After 1-2 spring injections of oxytetracycline (OX), kasugamycin (KS) and CC, we evaluated inoculated apple trees for blossom and shoot blight incidence. In a separate study, after spraying or injection of PC, we evaluated shoot blight severity after inoculation. At a high disease pressure, OX, KS and CC provided blossom blight control of 60.6, 31.7 and 15.5-17.8%, respectively. The same compounds provided control of shoot blight incidence of 60.7, 42 and 24.5-33.9%, respectively. The results indicate that shoots initially accumulate more of the injected compound than flowers, due to their higher transpiration driven by larger green tissue area. Sprayed PC reduced shoot blight severity for 25.6% and caused expected reduction of shoot length, while trunk-injected PC failed to cause any of these effects. This indicates that PC did not translocate into the canopy due to its strong binding in the xylem. With the development and use of injectable formulations, proper dosing, and optimal injection timing, delivered compounds could have more time for accumulation in the canopy and thus provide better fire blight control. Hence, trunk injection could become an effective option for fire blight control on apple trees.

Key words: *Erwinia amylovora,* apple fire blight control, trunk injection, bactericides, prohexadione-calcium.

INTRODUCTION

Management of fire blight (*Erwinia amylovora*) is dependent on preventive spray applications of copper and a limited number of antibiotics such as streptomycin, oxytetracycline (OX) and kasugamycin (KS) (McGhee and Sundin, 2011). However, spray applications of antibiotics can lead to environmental side-effects such as selection of *E. amylovora* strains resistant to antibiotics (McGhee and Sundin, 2011) and drift-driven losses of antibiotic spray solution of up to 44-71% (Steiner, 1969). It is indicated that when non-target bacteria in the environment are exposed to streptomycin, resistance genes can be selected within their populations and may be transferred to plant pathogenic bacteria such as *E. amylovora* (Sundin

et al., 1995). It is fearfully believed that off-target deposition of antibiotics to soil and environment could significantly expose various inhabiting bacteria, that are viewed as sources of antibiotic resistance genes important for clinical pathogens (McManus, 2014). Concerns for potential bridging of the gap between the environmental and human gut niches, via antibiotic resistant bacteria residing on the fresh plant produce, impose scrutiny on antibiotic use for plant protection (McManus, 2014). The risks for this bridging to occur are very low since the mechanisms for transferring genes for antibiotic resistance are distinct between human and plant pathogens (Sundin, 2002). Further, the quantity of applied antibiotics in plant protection is very low, amounting to 0.26% in comparison to food animal production use (McManus, 2014). Still, the associated environmental risks keep the use of antibiotics in agriculture under scrutiny and bring into question the means by which we deliver materials for fire blight control. This supports investigation of more environmentally friendly solutions for compound delivery such as stem injection (Düker and Kubiak, 2011).

Search for alternatives to antibiotics such as plant resistance activators and biological control agents gained momentum in the past two decades. The results with sprayed activators of systemic acquired resistance (SAR), such as acibenzolar-S-methyl, showed quite variable and unpredictable levels of fire blight control and need for frequent reapplication. However, research on more eco-friendly ways in delivering compounds for fire blight control, such as trunk injection, received little attention (Spitko, 2008; Düker and Kubiak, 2011). Trunk injection is a novel method for pesticide delivery in agriculture which utilizes xylem to translocate and distribute the compound into the canopy (Percival and Boyle, 2005). It is used for tree protection and nutrition in landscape tree care where tree sizes and vicinity of urban areas limit the use of spray pesticide applications. Our recent research on distribution of trunk-injected imidacloprid in apple trees and on control of apple scab and insect pests with injected pesticides showed very promising results (Aćimović et al., 2014; VanWoerkom et al., 2014; Wise et al., 2014). However, there is no research addressing injection of bactericides for fire blight control. Research with trunk-injected structural resistance activator prohexadione-calcium (PC), which inhibits gibberellin biosynthesis and thus reduces shoot growth and susceptibility to fire blight, has shown no effect on fire blight (Spitko, 2008). In contrary, injected prohexadione-carboxylic acid, the free acid of PC, provided 13.6-17.5% of blossom blight control on apple saplings (Düker and Kubiak, 2011). The goal of this study was to assess the performance of trunk-injected bactericides and sprayed and injected PC in control of fire blight on apple flowers and shoots. We hypothesized that significant control of fire blight can be achieved with 1-2 trunk injections of these compounds.

MATERIALS AND METHODS

We created four cardinally oriented injection ports per tree, positioned approximately 15 cm above the ground level, by drilling 25.4 mm into the xylem tissue and 9.5 mm in diameter, with a cordless 1500 rpm drill (Aćimović et al., 2014). Ports were sealed with plastic-silicone plugs (Fig. 1A, B) (Arborplug® no. 4, Arborjet Inc., USA) according to the manufacturer's instructions. We conducted compound applications using doses in Table 1.

Trunk injections of all compounds except oxytetracycline (OX) were conducted with Tree I.V. Micro-Infusion™ System (Fig. 1C). Tree IV allows fast injection of large solution volumes using 413.7 kPa of air pressure. Due to small solution volume, OX was injected with Quik-jet® micro-injection system (Fig. 1D) which relies on hand-generated pressure. Injection device needles were inserted through the septum in the

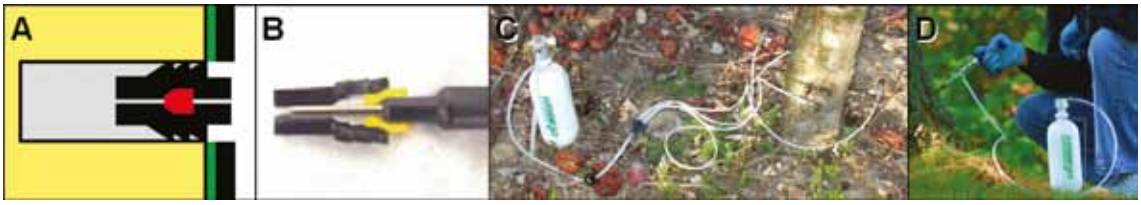

Figure 1. Arborplug® no. 4 (A) with silicone septum in the injection channel, acting as a one-way valve (red rectangle). Plug cross section showing needle penetrating the white septum during injection (B). Tree I.V.™ (C) and Quik-jet® (D) systems used to trunk-inject apple trees (Arborjet Inc., USA).

plugs thus allowing inflow of the protective solution into the ports (Fig. 1A, B). Total injected volume per tree was divided equally among the four ports. Sprayed PC was applied at a label rate for one topical treatment according to the EPA registration label in USA. Trees injected with 520 ml of water served as a control. Each treatment consisted of 4 replicate trees arranged in a completely randomized design (CRD).

For blossom and shoot blight incidence control on 21-yr-old 'Gala' apple trees, flowers were spray-inoculated with *E. amylovora* (0.7×10^6 CFU/ml) on 14 May 2013 at 80% bloom, using a hand sprayer (Solo 457, 11.36 L, Solo Inc., USA). Blossom and shoot blight incidences were evaluated in 7-day intervals. We randomly chose blossom clusters on spurs and counted the number of diseased and healthy clusters in a 100-cluster sample per tree. Blossom blight incidence was calculated as blossom blight percent on a per tree basis. After counting a 100-shoot per tree sample, we calculated shoot blight incidence by comparing numbers of randomly chosen blighted and healthy shoots for each tree. For each treatment, blossom and shoot blight incidence means were calculated from 4 replicate trees.

For shoot blight severity control, pathogen inoculations at petal fall were conducted on 7 May 2012 for 14-yr-old 'Gala', and on 10 June 2011 for 11-yr-old 'Jonathan' apple trees using 4.7×10^7 and 5×10^5 CFU/ml, respectively. The upper third of the leaf blade of the second or the third youngest leaf on the shoot tip were removed with scissors dipped in pathogen suspension. A total of 10 randomly chosen shoots per each tree were inoculated with *E. amylovora*, while additional 10 shoots on the same tree replicate were inoculated with distilled water as a negative control. For each inoculated shoot, severity was calculated from the ratio of necrotized shoot length and total shoot length (cm). Total shoot length (cm) was recorded for negative control shoots. Total shoot length was first taken prior to inoculation. Shoot and necrosis lengths were measured in 7-day intervals with cessation when the terminal bud set on shoots. Shoot blight severity mean per tree (%) was calculated from 10 shoot replicates. Average shoot blight severity in each treatment was calculated from 4 replicate tree means. From the severity data for each week, area under the disease progress curve (AUDPC) was calculated. All data were analyzed using SAS 9.3 (SAS Institute Inc., USA).

RESULTS

In 2013, injected OX, KS and copper chelate (CC) provided significant control of blossom blight incidence of 60.6, 31.7 and 15.5-17.8%, respectively

Table 1. Trunk-injected and sprayed compounds for fire blight control on apple trees

Treatment / active ingredient (product)		Dose	Date(s) of application
Injections for blossom and shoot blight incidence control on 'Gala' trees:			
Oxytetracycline hydrochloride 39.6% (ArborBiotic™)		0.31 g + 2.52 ml of water / 25.4 mm DFH [1]	1 May 2013
Kasugamycin hydrochloride 2.3% (Kasumin® 2L)		2 x 7.6 ml [2, 3]	1 and 22 May 2013
Copper chelate 1	water soluble copper 5% (Baicor® Cu)	2 x 5 ml / tree [3]	1 and 22 May 2013
Copper chelate 2		2 x 15 ml / tree [3]	
Treatments for shoot blight severity control on 'Gala' and 'Jonathan'** trees:*			
*Prohexadione-calcium - INJECTION	prohexadione-calcium 27.5% (Apogee®)	11.23 g / apple tree [3, 4]	23 April 2012
**Prohexadione-calcium - SPRAY		1360.8g / 0.405 ha + Regulaid® (125 ml /100 L) [5]	24 May 2011

[1]DHFH: trunk diameter at 15 cm height. [2]Dose for one spray according to the EPA registration label in USA, divided by 250 trees grown per 0.405 ha. [3]Dose injected with 520 ml of water per tree. [4]Maximum allowed seasonal dose per 0.405 ha according to the EPA registration label in USA. [5]Nonionic surfactant.

(Fig. 2A). The same compounds provided significant control of shoot blight incidence of 60.7, 42 and 24.5-33.9%, respectively (Fig. 2B). In 2011, sprayed PC reduced shoot blight severity for 25.6% (Fig. 2C) and caused the expected reduction of shoot growth (Fig. 3A), while none of these effects were replicated in 2012 with the injected PC (Fig. 2D and Fig. 3B).

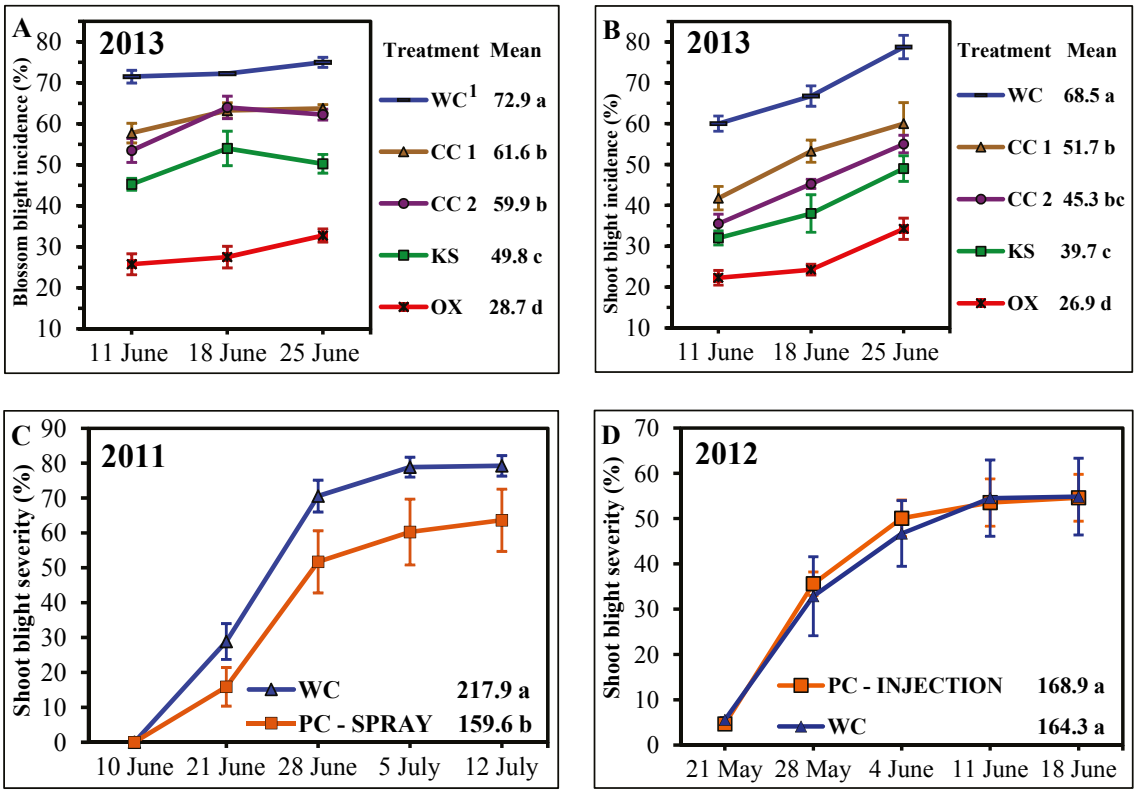

Figure 2. Fire blight control on 'Gala' apple trees with trunk injected bactericides (A, B), and with prohexadione-calcium sprayed on 'Jonathan' (C) and trunk-injected on 'Gala' trees (D). A, B: blossom and shoot blight incidence means across the three time points within one treatment followed by different letters are significantly different ($p<0.05$). C, D: mean values of Area Under the Disease Progress Curve (AUDPC) calculated from shoot blight severity followed by different letters are significantly different ($p<0.05$). [1]WC - water injected control, CC - copper chelate, KS - kasugamycin, OX - oxytetracycline, PC - prohexadione-calcium. Error bars represent standard error of the mean (SEM).

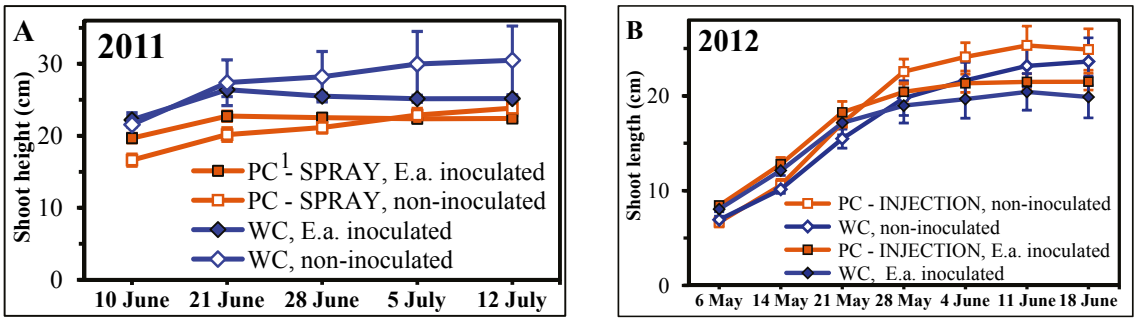

Figure 3. Shoot growth reduction after spraying of prohexadione-calcium on 'Jonathan' apple trees (A). Trunk-injected prohexadione-calcium caused no shoot growth reduction on 'Gala' trees (B). Shoot growth was monitored on the same trees from Fig. 2 C and D. [1]PC - prohexadione-calcium, WC - water injected control, E.a. - *Erwinia amylovora*. Error bars represent standard error of the mean (SEM).

DISCUSSION

Injected antibiotics provided best blossom and shoot blight control. Single injection of OX was better than the two injections of other bactericides. Depending on the study evaluating OX sprays, our injection of OX showed to be slightly or much better than the OX spray (Mycoshield®, 17% OX) which allowed blossom blight incidences of 29-37% or 57-67% (McManus and Jones, 1994; Stockwell et al., 2007). Thus, the injectable OX we used significantly reduced bacterial populations on flowers. It seems that injection is a superior delivery for OX, allowing its prolonged activity versus spraying, where OX levels quickly decline due to short half-life of OX and bacterial populations reestablish. Injected KS was not as good as its spray which reduced blossom blight to incidences of 1.5-5.6% (McGhee and Sundin, 2011). Significant fire blight reduction by injected CC is the first report of this copper form in fire blight control. It appears that injected bactericides weakly and slowly exude on the surface of flower stigmas, favorable for *E. amylovora* growth, but too late for better control effect. It seems that when pathogen invades inner flower tissues, the injected bactericides accumulated in the tissues only stop further pathogen dissemination on other flowers and invasion into the branches. The results on shoot blight incidence indicate that shoots initially accumulate more of the injected compound than flowers, due to their higher transpiration driven by larger green tissue area. This accumulation and thus control of fire blight later weakens due to dilution effect by increasing volume of green tissues.

Our results with injected PC align with the results on 'Paula Red' apple trees injected with PC, which showed no reduction of shoot blight and shoot growth (Spitko, 2008). PC probably did not translocate in xylem after injection and was bound in the trunk due to its high organic carbon-water partitioning coefficient (Koc) of 155-1428 ml/g and low water solubility of 174 mg/L (Serafini, 2001). High Koc depicts strong compound adsorption to carbon in a certain environment. PC spray gave control of shoot blight of 25.6% which was lower that the usually reported reduction of 40-86% (Momol et al., 1998). If xylem-mobile formulations of compounds are developed, and optimization of schedules and doses is conducted, trunk injection could be an effective option for eco-friendly fire blight control.

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INFLUENCE OF METEOROLOGICAL FACTORS ON THE OCCURRENCE OF FIRE BLIGHT SYMPTOMS IN DIFFERENT REGIONS OF MONTENEGRO

Dragana Radunović[1], Veljko Gavrilović[2] and Marija Krstić[3]

[1]Extension Service in Plant Production, Biotechnical Faculty, Podgorica, Montenegro
[2] Institute for Plant Protection and Environment, Belgrade, Serbia
[3]Ministry of Agriculture and Rural Development, Podgorica, Montenegro
draganaradunovic@t-com.me

INTRODUCTION

Since the first occurrence of fire blight caused by the bacterium *Erwinia amylovora*, it has been observed that the expression of symptoms and development of the disease depend on meteorological conditions, primarily temperature, air humidity and rainfall, during blooming period of susceptible fruit species. Weather conditions are likely the most important factor in epidemiology of fire blight (Van der Zwet and Keil, 1979).

Meteorological factors have a crucial impact on the occurrence and harmfulness of the disease during the critical blooming period of quince, apple and pear. Flowers are infected during warm days (temperature around 25^0C), which are followed by rainfall or high humidity (>70%), fog or dew. Temperatures lower than optimal in the blooming period significantly reduce population of this bacterium and inhibit the infection (Van der Zwet and Beer, 1995).

Expressive variations in specific local weather conditions between particular parts of Montenegro served as a good basis for monitoring the impact of basic meteorological factors on the occurrence of the symptoms and damage caused by this disease.

MATERIAL AND METHODS

During the spring of 2012 and 2013, the occurrence of fire blight symptoms was monitored on susceptible fruit species and spontaneous plant hosts of *E.amylovora* in different climatic regions of Montenegro. Monitoring included pome fruit orchards and individual pome fruit trees in the northern, central and southern parts of the country (Radunović and Gavrilović, 2013).

Meteorological data were simultaneously monitored using automated meteorological stations (METOS), placed in corresponding selected localities: Bijelo Polje (northeastern, mountainous region), Nikšić (western, mountainous region), Podgorica (central, lowland region) and Bar (southern, coastal region). The data were collected in April, May and June 2012 and 2013. Meteorological factors were examined, primarily the influence of temperature, relative air humidity and precipitation on manifestation of the disease symptoms, with a particular emphasis on monitoring the infection during the blooming phase.

Development phenophases of susceptible fruit species were also observed in the mentioned regions.

Obtained meteorological data (temperature, air humidity and precipitation amount), as well as the monitoring of phenophases in quince, apple and pear development, served as a basis for the use of a computer model „Cougarblight" (Smith, 1999) for forecasting the fire blight occurrence. This forecasting model provided information on the risks of infection (low, medium, high risk and the moment of infection) and expected appearance of symptoms on susceptible fruit species in the selected localities. Dates of favorable conditions for the infection were recorded. Attention was especially focused on monitoring the infection during the blooming phase, as crucial for the occurrence and spread of the disease.

RESULTS

Close correlation was determined between meteorological factors and the occurrence of fire blight symptoms in different regions of Montenegro.

Localities of B.Polje and Nikšić

It was determined by monitoring it's phenophases in mountain climate conditions of northeastern (locality of B.Polje) and western (locality of Nikšić) regions of the country, that quince blossomed approximately in the same period - from 2nd to 15th of May, with full blossoming around 9th of May.

In these localities, air humidity, recorded by monitoring of meteorological factors from the station, was constantly over 75% (in some days up to 99%) during this period. There were also rainy periods from 1st to 7th, and then from 9th to 14th of May, which means that it was raining during almost the whole period of quince blossoming. Average daily temperatures were between 17^{0}C and 18^{0}C, and during the four days of full quince blossoming they even reached 21^{0}C, which was around the lower limit of optimal values for the infection.

On the basis of these data, it was concluded that favorable meteorological parameters during quince blossoming: high relative air humidity (over 70%), longer rainy periods and temperatures within the optimum (21^{0}C), enabled mass infection of quince flowers in the localities of Nikšić and B.Polje.

Locality of Podgorica

In the central, lowland region of the country (locality of Podgorica), quince blossomed in the period from 14th to 26th of April, with full blossoming around 20th of April.

According to data from the station, there was no precipitation in the locality of Podgorica during the entire period of monitoring, except for one day (23rd of April).

During quince blossoming, air humidity did not exceed 40% and it was even below 30% for several days. Two-day exception with humidity of around 70% was on 23rd and 24th of April, when quince blossom was already fading. Temperatures were between 17^{0}C and 18^{0}C and they reached 21^{0}C towards the end of this period (24th and 25th of April), when quince blossom had already faded.

It was concluded that temperatures below lower limit of the optimum, lack or complete absence of precipitation and air humidity below 40% inhibited the infection of quince flowers in the locality of Podgorica. Optimal temperatures for the infection were reached only towards the end of the period, when quince blossom had already faded, which was, together with the other unfavorable factors, the reason why the infection did not occur.

Locality of Bar

In the southern, coastal region of the country (locality of Bar), quince blossomed from 11th to 23rd of April, with full blossoming around 17th of April. During the whole period, average daily temperatures were below optimal values for the infection (15^{0}C – 17^{0}C), air humidity was below 70% (50 – 70%) and there was no precipitation, except on 22nd and 23rd of April, when quince blossom had already faded. These parameters indicated unfavorable meteorological conditions, which inhibited the infection.

DISCUSSION

Intensive occurrence of *E.amylovora* on quince in northeastern (locality of B.Polje) and western (locality of Nikšić) regions of Montenegro, characterized by mountain climate, could be explained by coinciding of quince blooming period with longer rainy periods, followed by high air humidity and optimal temperatures for blossom infection. Mass infections of quince flowers resulted in rapid spread of this bacterium through shoots and thinner and thicker branches.

Lower infection intensity on apple and pear, recorded in the localities of B.Polje and Nikšić in spring 2012 and 2013, was connected with temperatures lower than optimal during the blooming phenophase of these fruit species and an intensive growth of their shoots.

In contrast to the northeastern and western regions, the disease symptoms were not observed on susceptible fruit species in the central, lowland region of the country (locality of Podgorica). Lack of rainfall and optimal temperatures during blooming of quince, pear and apple, in combination with low relative air humidity, which is climate characteristic of this region, proved to be limiting factors for the occurrence of the infection and symptoms of fire blight. Due to unfavorable meteorological factors, plants virtually "avoided" the infection during the blooming period - the most critical phase for the disease occurrence, which is why symptoms did not appear.

Considering that symptoms of fire blight were recorded in a smaller degree in only one pear crop in the southern, coastal region of the country (the locality of Bar), it was concluded that the bacterium was most likely transmitted by infected planting material, while unfavorable meteorological factors inhibited it's further spread and stronger infections.

Highly infected quince crops and single trees in the northeastern (locality of B. Polje) and western (locality of Nikšić) regions of the country, represented focal points from which this bacterium spread over new areas and new host plants.

Quince is considered as one of the most susceptible host of *E.amylovora* throughout the world (Arsenijević and Panić, 1996; Arsenijević and Gavrilović, 2007). First occurrence of the disease in Montenegro was recorded

also on quince, in 2003, in very high intensity (Obradović et al., 2003). Diseased quince trees are very important source of inoculum from further infection and spread of bacteria especially under favorable weather conditions (Balaž et al., 2012; Balaž et al., 2013).

Control of *E. amylovora* is very difficult due to lack of proper bactericides able to prevent spread of bacteria best efficiency showed antibiotics but they are not registered for applying for plant protecting in most of European country (Arsenijević, 1997; Gavrilović, 2009). Thus, prediction of occurrence disease is very important measure for pathogen control. Very important role in infection by *E. amylovora* is presence of epiphytic population of bacteria on fruit trees leaves and blossoms without obviosly symptoms (Thomson, 2000; Thurechek, 2004; Johnson et al, 2006).

Results obtained in this investigation could serve as a good reference when choosing localities for planting new crops of pome fruit species in Montenegro, all in order to prevent the occurrence and spread of fire blight.

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SPATIAL AND TEMPORAL DISTRIBUTION OF INSECT VECTORS OF *Xanthomonas campestris* pv. *musacearum* AND THEIR ACTIVITY ACROSS BANANA CULTIVARS GROWN IN RWANDA

Alexandre Rutikanga[1,3], Gertrude Night[2], Geofrey Tusiime[3], Walter Ocimati[4] and Guy Blomme[4]

[1] University of Rwanda and Bioversity International/CIALCA Project
P.o. Box 210 Musanze or P.o. Box 3971 Kigali (Kigali, Rwanda)
alexandrerut@yahoo.fr or rutikangaalexandre@gmail.com
[2] Rwanda Agriculture Board,
P. O Box 5016 (Kigali, Rwanda)
[3]Makerere University
P.O. Box 7062, (Kampala, Uganda)
[4] Bioversity International,
P.O. Box 24384, (Kampala, Uganda)

ABSTRACT

Insect vectors of *Xanthomonas campestris pv musacearum (Xcm)* have played a major role in long distance and plant to plant transmission of Xanthomonas wilt of banana (XW). The prevalence of insects has been reported to vary in space and time. Some banana cultivars have also been reported to attract more insect vectors of *Xcm* than others. The present study was conducted to determine the spatial and temporal distribution of insect vectors of *Xcm* and assess their activity across banana cultivars grown in Rwanda. The study was carried out in four banana growing areas of Rwanda selected on the basis of their altitude (i.e.Low, Medium and High). The Kivu Lake Border region was selected as a fourth site due to the high prevalence of XW. Insects were sampled in the four annual seasons (short dry, short rainy, long dry and long rainy) and at different times of the day. During sampling of insects, the incidence of XW-male bud infection was also recorded. Collected insects were immediately sorted into taxonomic groups and conserved in vials containing 70% ethanol for further identification to genus and species level. Five insect specimens in each taxon were put aside for the isolation of *Xcm* on their external body parts. There was a high prevalence of fruit flies, honey bees and other flies (in other families than Drosophilidae and Tephritidae) compared with wasps, ants and beetles. More insects were recorded in the low altitude area and during the long rainy season. These findings correlated with the observed high incidence of XW in the wet seasons. Incidence of floral infections was higher in the low altitudes declining with the increase in altitude, correlating with the decline in insect activity as altitude increased. The activity of insects on banana male buds varied among banana cultivars, with more activity on beer (AAA-East African Highland (EAH) and ABB types) and dessert banana cultivars compared with cooking or mixed use cultivars. Among the cooking types only 'Injagi' and its clone sets 'Barabeshya' and 'Incakara' attracted large insect populations. Banana cultivars 'Nkazikamwe' (cooking AAA-EAH), 'Impura' (beer AAA-EAH) and 'Ikinyangurube' (dessert AAA) possessed persistent male bracts and neuter flowers and were less attractive to flower visitors. These cultivars could be promoted in areas prone to insect vector infections. Timely and proper de-budding should be emphasized with special attention during the rainy seasons and for banana cultivars with non-persistent male buds.

Keywords: Xanthomonas wilt incidence, insect vectors, banana cultivars**,** growing areas and period

INTRODUCTION

Xanthomonas wilt of banana (XW) has become endemic in the east and central African region, since its first official reports in 2001 in the Democratic Republic of Congo and Uganda. The bacterial disease has drastically reduced banana productivity and is severely affecting farmer livelihoods (Carter *et al.,* 2010, Mbaka, *et al.,* 2009, Mgenzi *et al.,* 2006, Tushemereirwe et al., 2004; Mwangi, 2009; Ndungo et al, 2006) and (Reeder *et al*., 2007). No curative treatment has so far proven effective in controlling XW. Only cultural practices are recommended as preventive measures for the containment of the disease (Karamura *et al*., 2008). These are effective if sources of inoculum are removed and opportunities for spread are reduced or eliminated (Eden-Green, 2004 and Blomme *et al.,* 2005);). Insect vectors are one of the main mechanism of dispersal of *Xanthomonas campestris* pv. *musacearum* (*Xcm*), the causal agent of XW (Tinzaara *et al.,* 2006; Yirgou *et al*.; 1974;Yirgou *et al*., 1968). Insects are thought to play a major role in long distance and plant to plant transmission of the disease (Buregyeya *et al*., 2008; Fiaboe *et al*., 2008; Shimelash *et al.,* 2008). There is a symbiotic relationship between bananas and some insect species based on the fact that bananas serve as source of food (nectar and pollen) for insects and in return, insects act as the pollinators of bananas (Willson *et al.,* 1996). In the search for nectar, insects spread bacterial diseases from plant to plant. XW transmission by insect vectors is influenced by ecological conditions and bio-physical factors including the predominant cultivars grown. Ecological conditions greatly influence the life history and distribution of insect species. Climate conditions affect insect biology and behavior. These are dictated by variations in altitude whereby prevailing temperature, humidity and rainfall regime are determining factors in insect population fluctuations throughout the year (Salin *et al*., 1999).

For example, altitudes above 1,700 masl are reported to have less insect vector activity and thus lower floral XW infections (Shimelash *et al.,* 2008) than lower altitudes. Bio-physical conditions also dictate distribution of crop cultivars. This is the case in Rwanda where different banana (*Musa* spp.) cultivars grow across a range of altitudes with different climatic and soil conditions. The predominant banana cultivars in a cropping system greatly influence the extent of insect transmission of XW. For example, higher incidences of insect mediated floral XW infections have been reported in areas dominated by 'Pisang awak' (*Musa* ABB genome) in East Africa and Ethiopia (Addis *et al.,* 2004; Blomme *et al.,* 2005). In Uganda, predominant insect species reported on banana inflorescences include fruit flies (Drosophilidae), stingless bees (*Plebeina denotti*) and grass flies (Chloropidae). Based on field experiments, *P. denotti* and grass flies were reported to possibly spread XW from infected to healthy plants (Tinzaara *et al.,* 2006). The spread of XW is influenced by the predominant insect species. For example, fruit flies are present in large numbers but spend most of their lives on a few banana mats and thus spread the disease over short distances, whereas larger insects (e.g. bees) visit larger geographical areas/ numbers of flowering banana plants each day and can spread the disease over long distances. Understanding of the dynamics of insect species in different environments can improve our understanding of the dynamics of Xanthomonas wilt. This would in turn help improve management of the disease. The present study aimed at investigating the spatial and temporal distribution of insect vectors of *Xcm* and their activity on inflorescences of different banana cultivars grown in Rwanda.

MATERIALS AND METHODS

This study was conducted in major banana growing areas as distributed across the three main ranges of altitude prevailing in Rwanda: (i) low (800- 1,400 masl), (ii) medium (1,450-1,650 masl) and (iii) high altitude (1,700-2,200 masl) (Table 1). Districts touching on Lake Kivu borders were given special attention due to high XW infection that has devastated bananas in the area. The Lake Kivu border has a medium altitude in the range of (1,410-1,647 masl) but a higher rainfall and somewhat different soils compared with the three districts in the other group within the medium altitudes (Table 1). Across the growing areas, increasing altitude is associated with increased annual rainfall, lower annual temperatures, higher humidity and different soil groups (Table 1). The study covered the four annual seasons in Rwanda during 2012. Data were collected once per week for four weeks of the typical month characteristic of the season.

For the short dry season (January-February) sampling was done in January, for the short rainy season (March-May), it was done in April, for the long dry season (June-August), it was done in July and for the long rainy season (September-December), the sampling was done in October. In each altitude category, three districts with Xanthomonas Wilt disease of banana (XW) were selected and a highly infected Sector (administrative division below District) chosen for data collection (Table 1). Four XW-infected banana fields were selected in each Sector for the collection of insects from three plants per banana cultivar selected based on cultivar availability in each field. It is worthy to note that different plants

of the same cultivar, preferably on the same mat/stool, were considered for each sampling time. Sweep nets were used to capture flying insects on the male inflorescence of individual plants. Other insects such as ants and small beetles were handpicked and put into vials. It is important to note that separate nets were used to collect flying insects that were used for *Xcm* isolation so as to avoid *Xcm* contamination among insects from different taxa.

This was done by waving insect nets in the air around male flowers of individual plants. For this purpose, bees, flies and wasps were scared off the male buds of the plants by a simple touch and individual insects were trapped into the net as they tried to escape. In contrast, fruit flies did not fly far away from the floral parts and were easily trapped by shaking them off into the net. To understand the distribution of insect species during the day, insect sampling was done on the same day between: i) 7 and 9 am; ii) 10 am and 12 noon; iii) 1 and 3 pm and iv) 4 and 6 pm. The weather conditions at the time of sampling were also noted and sampling during extreme weather conditions such as during or soon after rains was avoided. Captured insects were immediately sorted into their respective taxonomical orders, counted and put into labeled vials containing 70% ethanol for further identification to family and species level. Identification to family level was performed in the entomology laboratory of the Higher Institute of Agriculture and Animal Husbandry (ISAE-Busogo) in Rwanda. The identification exercise was supported by use of electron microscope and a data base for insect species available on the internet (i.e. Canadian Journal of Arthropod Identification 1911-2173; http:// bugguide. net/node/view and Castner *et al.,* 2000). Five specimens of insects that were morphologically similar (e.g. five bees of the same species) were put together in vials for isolation of *Xcm* from their external body parts.

Bacterial isolation was done by washing the insect body with distilled water. Ten µL of the resulting suspension was cultured by spreading in a petri-dish containing a semi-selective medium of Cellobiose Cephalexin Agar composed of yeast extract (1 gL^{-1}), glucose (1 gL^{-1}), peptone (1 gL^{-1}), NH_4Cl (1 gL^{-1}), $MgSO_4.7H_2O$ (1 gL^{-1}), K_2HPO_4 (3 gL^{-1}), beef extract (1 gL^{-1}), cellobiose (10 gL^{-1}), agar (14 gL^{-1}), cephalaxin (40 mg), 5-fluorouracil (10 mg) and cycloheximide (120 mg). Cultures were incubated for a period of 72 hours at 25°C (Mwangi *et al.,* 2007; Tripathi *et al.,* 2007). The resulting colonies were compared with pure cultures isolated from *Xcm*-infected banana plants and used in pathogenicity tests that were performed by inoculating healthy banana tissue culture plantlets. In the fields selected for sampling, the morphology of the male bud (persistence/non-persistence of the male bud bracts) and XW incidence of plants showing signs characteristic of insect vector transmission (i.e. wilting of the male bud) on three plants, purposively selected per cultivar, were also recorded. The data (for XW incidence) were square root transformed; GenStat 11th Edition (VSN International Ltd, 2008) statistical package used to generate the Analysis of variance (ANOVA) and the means separated using the Least Significant Difference at 5%.

RESULTS

Investigations on the current banana cultivars, their distribution and the prevalence of XW-floral infection across the study areas formed a basis to determine the spatial and temporal distribution of insect vectors of *Xcm*.

Table 1. Description of the study areas

Category of altitude	District	Sector	Altitude (masl)	Type of soil	Annual rainfall (mm)	Mean Annual temperature (°C)	Relative Humidity (%)
Low	1.Kayonza	Mukarange	1,300	Ferrisols	800-1,000	25	60
	2.Gatsibo	Kiziguro	1,400	Ferrisols	800-1,000	24	70
	3.Nyagatare	Tabagwe	1,380	Ferrisols	800-1,100	25	50
Medium	1.Ruhango	Bunyogombe	1,600	Granites	1,000-1,100	21	70
	2.Huye	Mukura	1,650	Paragneiss	1,000-1,100	20	80
	3.Gisagara	Save	1,600	Orthogneis	1,000-1,100	20	75
High	1.Rulindo	Rusiga	1,887	Granites	1,680-1,970	17	90
	2.Gakenke	Nemba	2,112	Granites	1,700-2,200	16	92
	3.Burera	Kinoni	2,167	Volcanic	1,800-2,500	13.2	95
Kivu lake border (Medium)	1.Rubavu	Rugerero	1,600	Volcanic	1,200-1,350	21	75
	2.Rutsiro	Mushyonyi	1,642	Granites	1,200-1,350	20	85
	3.Karongi	Bwishyura	1,596	Ferrisols	1,200-1,350	20	80

Banana cultivar distribution in the study areas

A total of 27 banana cultivars grouped into four categories (based on their main end use); dessert, beer, cooking and multipurpose bananas, were identified in this study (Table 2). The most dominant banana cultivars across all the study sites were the cooking (41%) and the beer (33%) banana cultivars, while the dessert and multipurpose banana cultivars represented only 19% and 7% of the cultivars, respectively (Table 3). Most of the cooking (45%) banana cultivars were observed in the low altitudes zone. The cultivars in the other use groups were more or less evenly distributed across the altitudes (Table 2, Table 3). Though the dessert banana cultivars 'Kamaramasenge' elsewhere known as 'Sukali ndizi', 'Gros Michel', 'Igisukari'; and the beer cultivar 'Kayinja' (Pisang awak) and other two cultivars ('Kivuvu' (Bluggoe) and 'Gisubi') in the same clone set were recorded from all the studied altitudes, their occurrence was sporadic. The beer bananas, 'Impura' and 'Umuzibo' both belonging to the *Musa* AAA genotype were found exclusively grown in the Lake Kivu Border region (medium altitude), while the beer banana 'Nyiramabuye' (*Musa* AAA) was only grown in Ruhango District (medium altitude). The beer bananas 'Ingumba', 'Ingenge' and 'Ingaju' were only found

Table 2. Distribution of banana cultivars in the study areas and their description based on vernacular names, synonyms, the morphology of the male bud, genome group and the main use. Abbreviations for districts are: Kay: Kayonza, Gat: Gatsibo, Nya: Nyagatare, Ruh: Ruhango, Gis: Gisagara, Huy: Huye, Rub: Rubavu, Rut: Rutsiro, Kar: Karongi, Rul: Rulindo, Gak: Gakenke, Bur: Burera. The signs '+' and '-' means the presence and the absence of the banana variety, respectively, in the study area.

Description of banana cultivars					Distribution of banana cultivars in the study areas										
Cultivar	Synonyms (farmers)	Morphology of male bud	Genome group	Main Use	Low altitude			Medium altitude			Kivu Lake border			High altitude	
					Kay	Gat	Nya	Ruh	Gis	Huy	Rub	Rut	Kar	Rul	Gak	Bur
'Barabeshya'	'Injagi', 'Incakara'	Not persistent	AAA	Cooking	-	-	-	+	+	+	+	+	+	+	+	+
'FHIA17'		Not persistent	AAAA	Multiple	+	+	+	+	+	+	+	+	+	+	+	+
'FHIA25'		Not persistent	AAB	Multiple	+	+	+	+	+	+	+	+	+	+	+	+
'Gros Michel'	'Mbogoya'	Not persistent	AAA	Dessert	+	+	+	+	+	+	+	+	+	+	+	+
'Igisukari'		Not persistent	AAA	Dessert	+	+	+	+	+	+	+	+	+	+	+	+
'Ikinyangurube'	Dwarf Cavendish	Persistent	AAA	Dessert	-	-	-	-	-	-	+	+	+	-	-	-
'Impura'	'Bakenga'	Persistent	AAA	Beer	-	-	-	-	-	-	+	+	+	-	-	-
'Incakara'	'Injagi', 'Barabeshya' Impundahunde	Not persistent	AAA	Cooking	-	-	-	-	-	-	+	+	+	-	-	-
'Ingagara'		Not persistent	AAA	Cooking	+	+	+	-	-	-	-	-	-	-	-	-
'Ingaju'		Not persistent	AAA	Beer	+	+	+	-	-	-	-	-	-	-	-	-
'Ingame'	'Indaya', 'Yangambi Km5'	Not persistent	AAA	Beer	-	-	-	+	+	+	+	+	+	+	+	+
'Ingenge'		Not persistent	AAA	Beer	+	+	+	-	-	-	-	-	-	-	-	-
'Ingenge'		Not persistent	AAA	Cooking	+	+	+	-	-	-	-	-	-	-	-	-
'Ingumba'		Not persistent	AAA	Beer	+	+	+	-	-	-	-	-	-	-	-	-
'Injagi'	'Incakara','Barabeshya'	Not persistent	AAA	Cooking	+	+	+	-	-	-	+	+	+	-	-	-
'Intokatoke'	'Inyarwanda'	Not persistent	AAA	Cooking	+	+	+	+	+	+	+	+	+	+	+	+
'Intuntu'		Not persistent	AAA	Beer	+	+	+	+	+	+	+	+	+	+	+	+
'Intusi'		Not persistent	AAA	Cooking	+	+	+	-	-	-	-	-	-	-	-	-
'Inzirabahima'		Not persistent	AAA	Cooking	+	+	+	-	-	-	-	-	-	-	-	-
'Inzirabushera'		Not persistent	AAA	Cooking	+	+	+	-	-	-	-	-	-	-	-	-
'Kamaramasenge'	'Kamara', Sukali ndizi	Not persistent	AAB	Dessert	+	+	+	+	+	+	+	+	+	+	+	+
'Kayinja' (Pisang awak)	'Kivuvu'(Bluggoe), 'Gisubi'	Not persistent	ABB	Beer	+	+	+	+	+	+	+	+	+	+	+	+
'Kibuzi'		Not persistent	AAA	Cooking	+	+	+	-	-	-	-	-	-	-	-	-
'Nkazikamwe'	'Mbwazirume', 'Kiryumukungu'	Persistent	AAA	Cooking	+	+	+	+	+	+	+	+	+	+	+	+
'Nyiramabuye'		Not persistent	AAA	Beer	-	-	-	+	-	-	-	-	-	-	-	-
'Poyo'	'Cavendish'	Not persistent	AAA	Dessert	+	+	+	+	+	+	+	+	+	+	+	+
'Umuzibo'		Not persistent	AAA	Beer	-	-	-	-	-	-	+	+	+	-	-	-

Table 3. Distribution of banana cultivars across the districts based on end use (dessert, beer, cooking and multipurpose) and distribution among altitude groups.

Banana Cultivars groups	Distribution across all districts (%)	Distribution per altitude group (%)			
		Low altitude	Medium altitude	High altitude	Lake Kivu Border
Dessert	19	25	25	25	25
Beer	33	29	24	18	29
Cooking	41	45	15	15	25
Multipurpose	7	25	25	25	25

grown in the low altitude region. The cooking banana cultivars 'Ingenge', Inzirabushera', 'Inzirabahima', 'Intutsi', 'Kibuzi' and 'Ingagara' were also only observed in the low altitudes. Of all the 27 banana cultivars, only three cultivars 'Impura' (also known as 'Bakenga'), 'Ikinyangurube' and 'Nkazikamwe' (elsewhere known as 'Mbwazirume') had persistent male bud bracts (Table 2). All the banana cultivars sampled except 'Kamaramasenge' (*Musa* AAB), FHIA25 (*Musa* AAB), FHIA (*Musa* AAAA) and 'Kayinja' (*Musa* ABB) belonged to the *Musa* AAA genomic group (Table 2).

Incidence of XW-floral infection in the study areas

The incidence of Xanthomonas Wilt of banana based on floral symptoms varied with the altitude, season and cultivars. However, no case of XW-male bud infection was recorded in the high altitude. Relatively higher incidences were recorded in Lake Kivu Border region and in the low altitudes. The incidence seemed to vary with cultivar distribution within each study area (agro-ecology). In the Lake Kivu Border region, the beer banana 'Ingame' (*Musa* AAA) elsewhere known as 'Yangambi Km5, showed the highest (5.66%) XW-male bud infection.

Other highly susceptible banana cultivars in this area were the beer banana 'Kayinja' (*Musa* ABB), the dessert bananas 'Kamaramasenge' (*Musa* AAB) and 'Poyo' (*Musa* AAA), and the cooking banana 'Incakara' (*Musa* AAA). In the low altitude, the beer banana 'Ingumba' (*Musa* AAA) was recorded with the highest incidence (5.79%) of male bud infection (Table 4). Xanthomonas Wilt-male bud infection was recorded across all annual seasons with higher prevalence observed during the rainy seasons.

Table 4. Incidence of XW-floral infection (square root transformed data) across major banana cultivars grown in Rwanda, respective cultivar genomes and end use in the four agro-ecologies and during the four annual seasons: Long dry Season (LdS), Long rainy Season (LrS), Short dry Season (SdS) and Short rainy Season (SrS).: Low altitude (Low), Medium, High altitude (High), and Lake Kivu Border (LKB) with altitude (Medium). Means with same letter within a row are not significantly different at P< 0.05.

Cultivar	Genome	Use	XW incidence (%) across agro-ecologies				XW incidence (%) across annual seasons			
			Low	Medium	High	LKB	LdS	LrS	SdS	SrS
'Barabeshya'	AAA	Cooking	-	2.00b	0a	0a	0a	1.17b	0a	0.83b
'FHIA17'	AAAA	Multipurpose	2.83b	0a	0a	0a	0a	1.17b	0.83b	0.83b
'FHIA25'	AAB	Multipurpose	0.83a	0a	0a	0b	0a	0.83b	0a	0a
'Gros Michel'	AAA	Dessert	2.83b	0a	0a	4.27c	0a	2.83c	2.00b	2.27b
'Igisukari'	AAA	Dessert	2.49b	0a	0a	2.49b	0a	1.66b	1.66b	1.66b
'Ikinyangurube'	AAA	Dessert	-	-	-	0	0a	0a	0a	0a
'Impura'	AAA	Beer	-	-	-	0	0a	0a	0a	0a
'Incakara'	AAA	Cooking	-	-	0a	5.10b	0.83a	1.66b	1.17a	1.44b
'Ingagara'	AAA	Cooking	4.27	-	-	-	0.83a	1.44b	0.83a	1.17ab
'Ingaju'	AAA	Beer	1.66	-	-	-	0a	0.83b	0a	0.83b
'Ingame'	AAA	Beer	-	1.65b	0a	5.66c	1.17a	2.49b	1.17a	2.49b
'Ingenge'	AAA	Beer	3.59	-	-	-	1.33a	1.17b	0.83ab	0.83b
'Ingenge'	AAA	Cooking	5.39	-	-	-	0a	1.44b	1.17b	1.44b
'Ingumba'	AAA	Beer	5.79	-	-	-	0.82a	1.87b	1.44b	1.66b
'Injagi'	AAA	Cooking	5.07	-	-	3.31b	0.82a	2.00b	0.83a	1.66b
'Intokatoke'	AAA	Cooking	2.00a	-	-	-	0.83a	1.66b	1.17b	1.44b
'Intuntu'	AAA	Beer	4.00b	0a	0a	4.95c	2.00a	2.61b	2.00a	2.35ab
'Intutsi'	AAA	Cooking	4.87	-	-	-	0.83a	1.44b	1.17ab	1.44b
'Inzirabahima'	AAA	Cooking	2.49	-	-	-	0a	0.83b	0.83b	0.83b
'Inzirabushera'	AAA	Cooking	2.00	-	-	-	0a	1.00c	0.42ab	0.83bc
'Kamaramasenge'	AAB	Dessert	5.31c	1.66b	0a	5.10c	1.66a	4.36d	2.35b	3.70c
'Kayinja'	ABB	Beer	5.10c	2.49b	0a	5.10c	1.66a	4.15d	3.174b	3.701c
'Kibuzi'	AAA	Cooking	3.31	-	-	-	0.83a	0.83a	0.83a	0.83a
'Nkazikamwe'	AAA	Cooking	0a	0a	0a	0.81b	0a	0a	0.83b	0a
'Nyiramabuye'	AAA	Beer	-	4.92	-	-	0a	2.05b	1.44b	1.44b
'Poyo'	AAA	Dessert	3.44c	0.83b	0a	5.18d	1.17a	3.92c	2.00b	2.35b
'Umuzibo'	AAA	Beer	-	-	0a	3.44b	0a	1.44c	0.83b	1.17bc
LSD						0.27				0.55
Cv%						86.5				17.8

The highest incidences (4.36% & 4.15% for 'Kamaramasenge' and 'Kayinja', respectively) were recorded during the long rainy season (LrS). Second to this were incidence levels noted during the short rainy season (SrS) (3.70) for both the varieties 'Kamaramasenge' (*Musa* AAB) and 'Kayinja' (*Musa* ABB), respectively (Table 4).

Identification of insect species visiting banana male buds and assessment of their ability to carry Xcm.

Seventeen insect species were collected across the different banana cultivars grown in the different agro-ecologies. Of these, there was two species of bees (Hymenoptera: Apidae), nine species of flies (Diptera: Drosophilidae, Tephritidae , Lonchaedae, Muscidae, Neriidae and Sarcophagidae), two species of wasps (Hymenoptera: Vespidae), three species of beetles (Coleoptera: Nitudulidae, Tenebrionidae, Staphylinidae) and one species of ant (Hymenoptera: Formicidae) (Table 5). *Xanthomonas campestris* pv. *musacearum* was isolated from the external body parts of all groups of insects collected from banana plants.. Light yellow, mucoid bacterial colonies characteristic of *Xcm* were observed to grow on culture media after plating 10 µl of suspension from insect bodies on a semi-selective media. However, fewer ants and beetles compared with the other insect groups carried the bacteria (Table 5). It is worthy to note that 'Fruit fly' will be used here as a common name for flies in the families 'Tephritidae and Drosophilidae' while other flies will indicate true flies in the families other than Tephritidae and Drosophilidae.

Activity of insect species across agro-ecological zones

All the identified insect species were observed across all the study areas. However, their prevalence differed. More insects were collected from the low altitude and the Lake Kivu Border region than in mid and high altitude zones (Figures 1-8). Among them the fruit flies dominated. Bee species (Hymenoptera: Apidae) came in second position in terms of number while the true fly species took the third position. Wasps, ants and beetles were present in small numbers and the trend for the proportion of insect prevalence was almost the same across all the study areas (Figures 1-8).

Table 5. Taxonomical identification of collected insects and the proportion (%) of them that carried *Xcm* on their external body parts in relation to the season of the year when they were captured.

Taxonomical Identification				Proportion (%) of typical colonies of Xcm isolated from external body parts of insects			
Common name	Order	Family	Species	Short dry	Short rainy	Long dry	Long rainy
1.Fruit fly	Diptera	Tephritidae	*Ceratitis rosaKarsch*	40±3.4	60±3.5	20±3.2	80±2.8
2.Bee	Hymenoptera	Apidae	*Apis sp*	40±3.4	60±3.5	20±3.2	80±2.8
3.Bee	Hymenoptera	Apidae	**NI*	20±3.4	40±3.5	40±3.2	100±2.8
4. Fruit fly	Diptera	Drosophilidae	*Drosophila sp.*	40±3.4	60±3.5	20±3.2	80±2.8
5. Fruit fly	Diptera	Drosophilidae	*Zaprionus sp.*	60±3.4	20±3.5	40±3.2	100±2.8
6. Fruit fly	Diptera	Drosophilidae	*Leucophanga sp.*	40±3.4	40±3.5	20±3.2	60±2.8
7.Fly	Diptera	Lonchaedae	*Silba sp.*	20±3.4	60±3.5	40±3.2	80±2.8
8.Fly	Diptera	Muscidae	*Neomyia rudissima (Loew)*	40±3.4	40±3.5	40±3.2	100±2.8
9.Fly	Diptera	Neriidae	*Carpophilus sp.*	20±3.4	20±3.5	40±3.2	80±2.8
10.Fly	Diptera	Sarcophagidae	*Sarcophaga sp.*	40±3.4	40±3.5	20±3.2	60±2.8
11.Fly	Diptera	Muscidae	*Musca domestica*	40±3.4	60±3.5	20±3.2	80±2.8
12.Wasps	Hymenoptera	Vespidae	*NI*	20±3.4	20±3.5	40±3.2	40±2.8
13.Wasps	Hymenoptera	Vespidae	*NI*	20±3.4	0±3.5	40±3.2	60±2.8
14.Ant	Hymenoptera	Formicidae	*NI*	20±3.4	20±3.5	0±3.2	20±2.8
15.Beetle	Coleoptera	Nitidulidae	*NI*	0±3.4	20±3.5	20±3.2	20±2.8
16.Beetle	Coleoptera	Tenebrionidae	*NI*	20±3.4	20±3.5	0±3.2	20±2.8
17.Beetle	Coleoptera	Staphylinidae	*NI*	20±3.4	0±3.5	20±3.2	20±2.8
Average				29.4±3.4	34.1±3.5	25.9±3.2	63.5±2.8

**NI: Not identified*

Variation of insect activity with time of the day

A highly significant difference ($P < 0.001$) was observed in the population of insect species collected throughout the four times of day and in all the three altitude zones (Figures 1 – 4). In all altitudes, fruit flies were more dominant, followed by bees and other flies (Figures 1 – 4). The other insects (wasps, ants and beetles) were relatively less prevalent and active on banana inflorescences in all surveyed areas in Rwanda (Figures 1- 4). Fruit flies were more prevalent in the morning hours (7 am to 9 am),

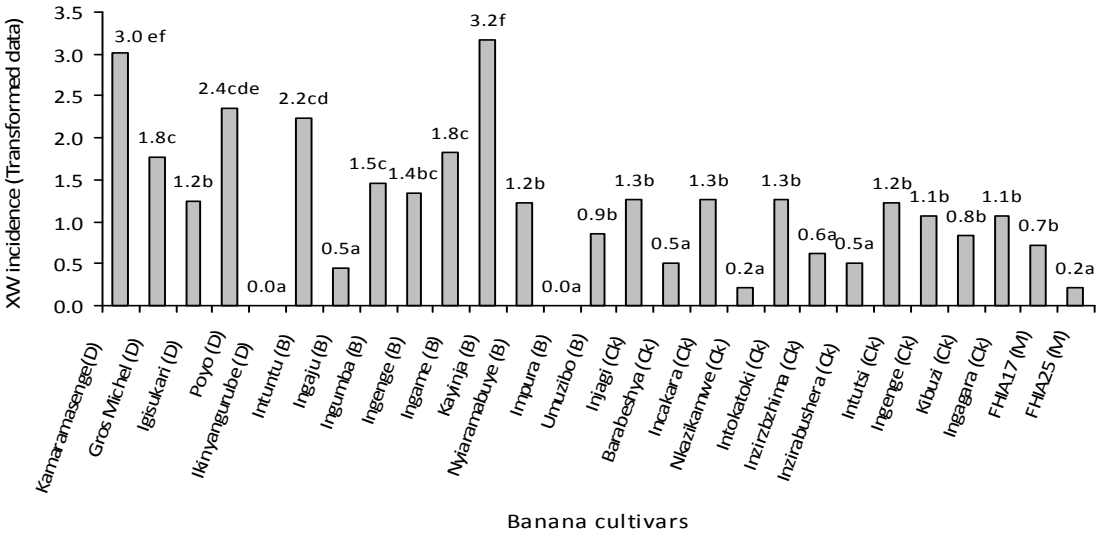

Figure 1. Xanthomonas wilt (XW) incidence (square root transformed data) with male bud infection across banana cultivars grown in Rwanda. Means followed by the same letter are not significantly different at $p \leq 0.05$

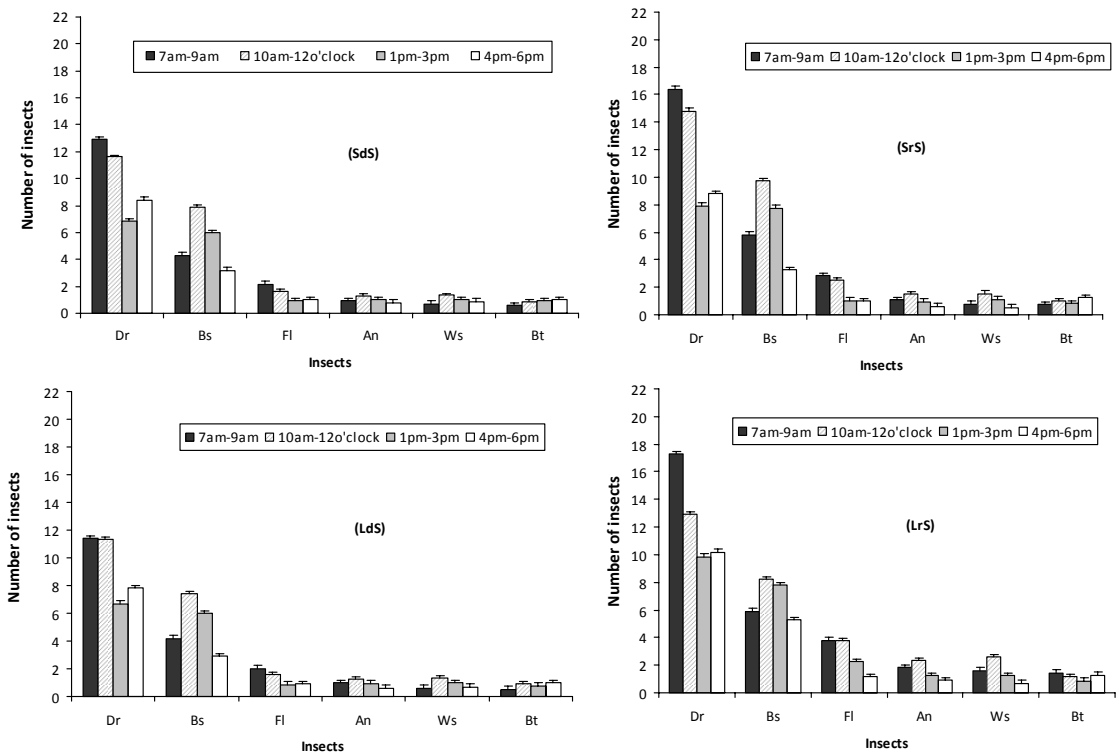

Figure 2. Insect activity during different time of the day in the High Altitude zone during the Short dry Season (SdS), Short rainy Season (SrS), Long dry Season (LdS).Error bars indicate the SEM. Letters at the 'X' axis stand for: (Dr) Drosophilidae, (Bs) Bees, (Fl) Flies, (An) Ants, (Ws) Wasps and Beetles (Bt). The error bars indicate the Standard Error of Means

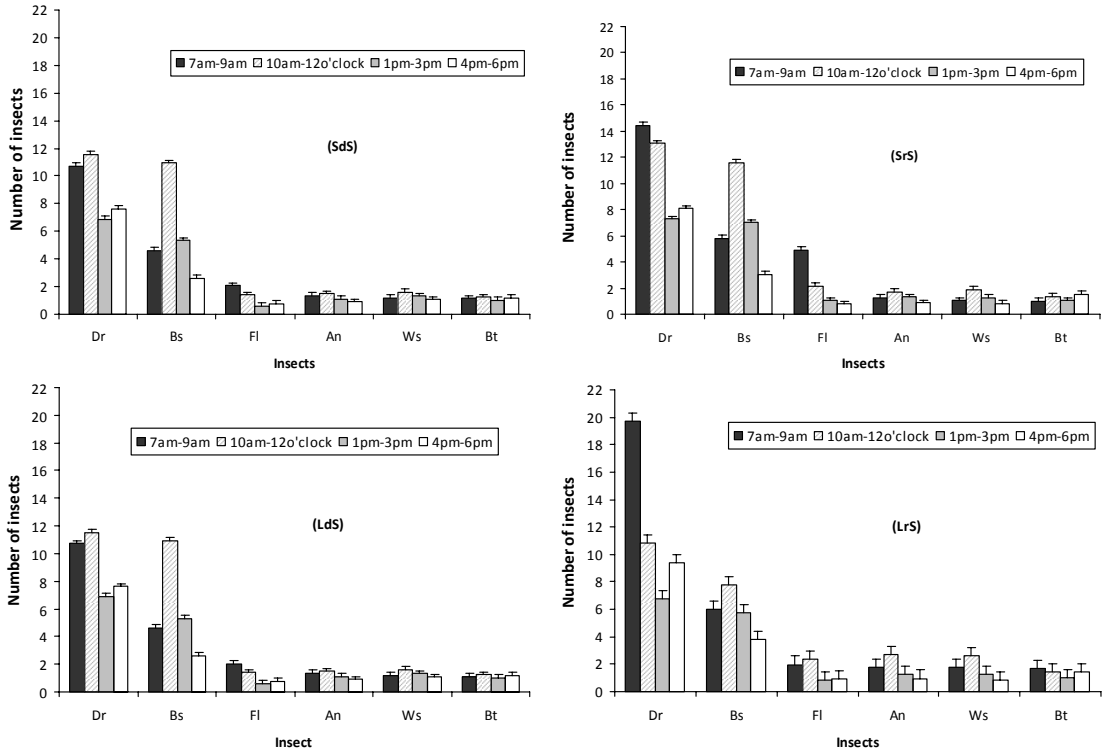

Figure 3. Insect activity during different time of the day in the Medium Altitude during the Short dry Season (SdS), Short rainy Season (SrS), Long dry Season (LdS) and the Long rainy Season (LrS). Error bars indicate the SEM. Letters at the 'X' axis stand for: (Dr) Drosophilidae, (Bs) Bees, (Fl) Flies, (An) Ants, (Ws) Wasps and Beetles (Bt). The error bars indicate the Standard Error of Means

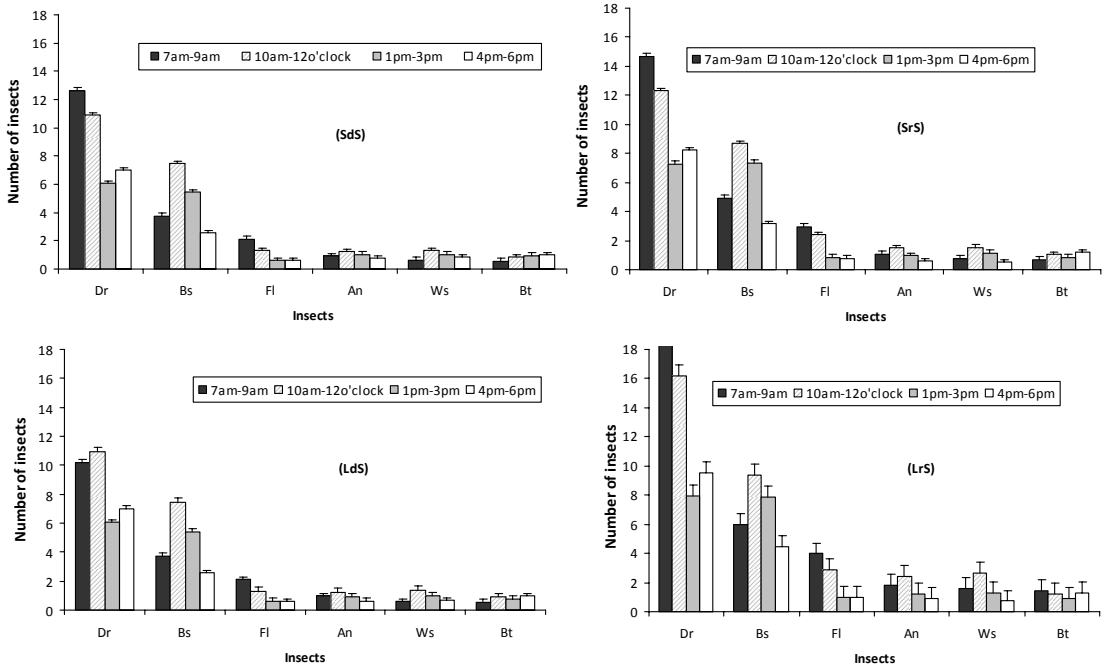

Figure 4. Insect activity during different time of the day in the Kivu Lake Border during the Short dry Season (SdS), Short rainy Season (SrS), Long dry Season (LdS) and the Long rainy Season (LrS). Error bars indicate the SEM. Letters at the 'X' axis stand for: (Dr) Drosophilidae, (Bs) Bees, (Fl) Flies, (An) Ants, (Ws) Wasps, (Bt) Beetles.The error bars indicate the Standard Error of Means

declining from mid-morning (10 am) to the late afternoon (3 pm) and rising slightly in the evening (4 pm to 6 pm) (Figures 1- 4). Higher populations of flies were also recorded in the morning, declining during the day. This trend was consistent in all agro-ecologies and seasons (Figures 1 – 4).

However, the difference between the number of flies captured in the morning and in the period ranging from mid-morning to mid-day was not significantly different (P< 0.005). Similarly, no significant differences were noted between numbers of flies collected in the afternoon (1 pm-3 pm) or in the evening (4pm-6pm) (Figures 1- 4). Bees were less active on banana inflorescences in the morning (7 am – 9 am), peaking between 10 am and 12 noon, and declining towards the evening (Figures 1 – 4). The average number of wasps, ants and beetles per banana plant was in general relatively low across all altitudes (Figures 1 – 4). Wasps and ants were observed to be higher in the morning (7 am-9 am) while beetles were more active during the morning and evening (7 am-9 am and 4 pm-6 pm) (Figures 1-4).

Activity of insect species across seasons

Prevalence of insects was significantly different (P < 0.001) between the annual seasons. Lower insect numbers were recorded in the long dry season, while the largest number occurred during the long rainy season (Figures 1 –8). During rainy days the population of ants doubled compared with fine days with excessively high activity during early and late evening. Flies were also more active during rainy days compared to sunny days. No correlation was observed between bee activity and weather patterns. Similarly the activity of beetles was consistent across seasons.

Activity of insect species on banana cultivars

Insect activity (based on the population of insect species collected) on male buds of different banana cultivars (dessert, beer, cooking and multipurpose) grown in the four agro-ecologies of Rwanda, differed significantly (P<.001). Beer and dessert banana cultivars in general attracted more insects compared with cooking and multipurpose banana genotypes (Figures 5-8). The beer banana 'Kayinja' (*Musa* ABB), the dessert banana 'Kamaramasenge' (*Musa* AAB) and the East African Highland cooking banana 'Injagi' (*Musa* AAA), were the leading cultivars in attracting insects within their respective use groups and this was consistent in all altitudes (Figures 5- 8).

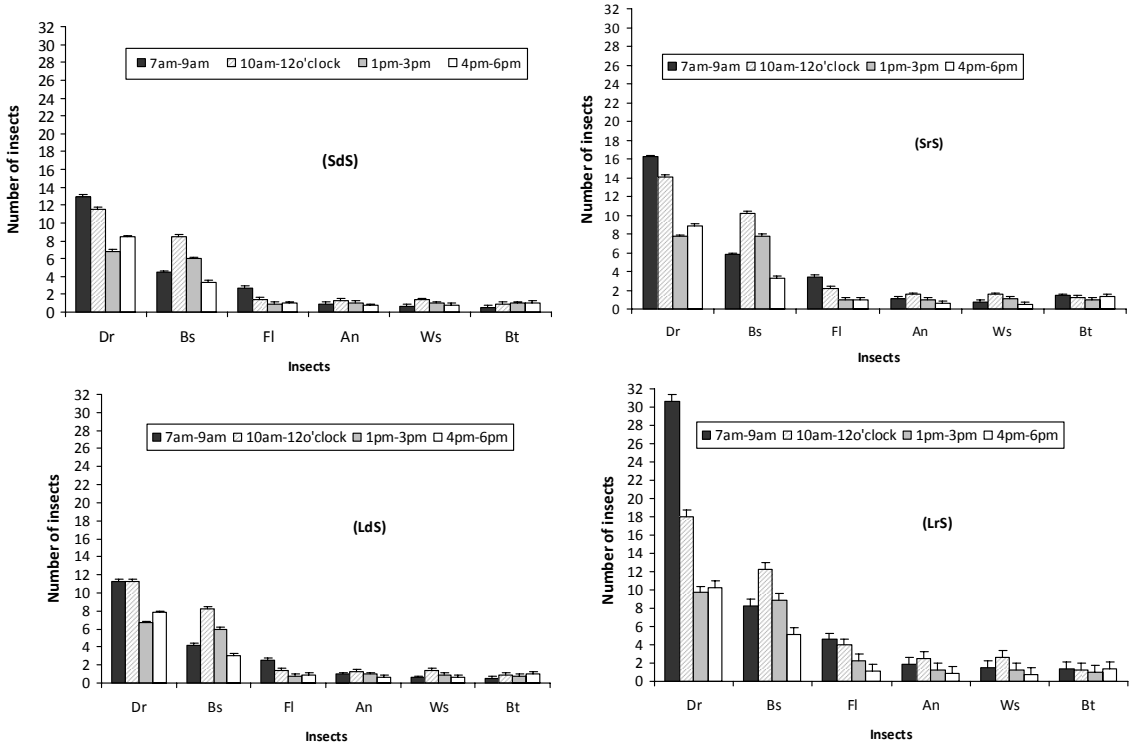

Figure 5. Insect activity during different time of the day in the Low altitude during the Short dry Season (SdS), Short rainy Season (SrS), Long dry Season (LdS) and the Long rainy Season (LrS). Error bars indicate the SEM. Letters at the 'X' axis stand for: (Dr) Drosophilidae, (Bs) Bees, (Fl) Flies, (An) Ants, (Ws) Wasps, (Bt) Beetles. The error bars indicate the Standard error of Means

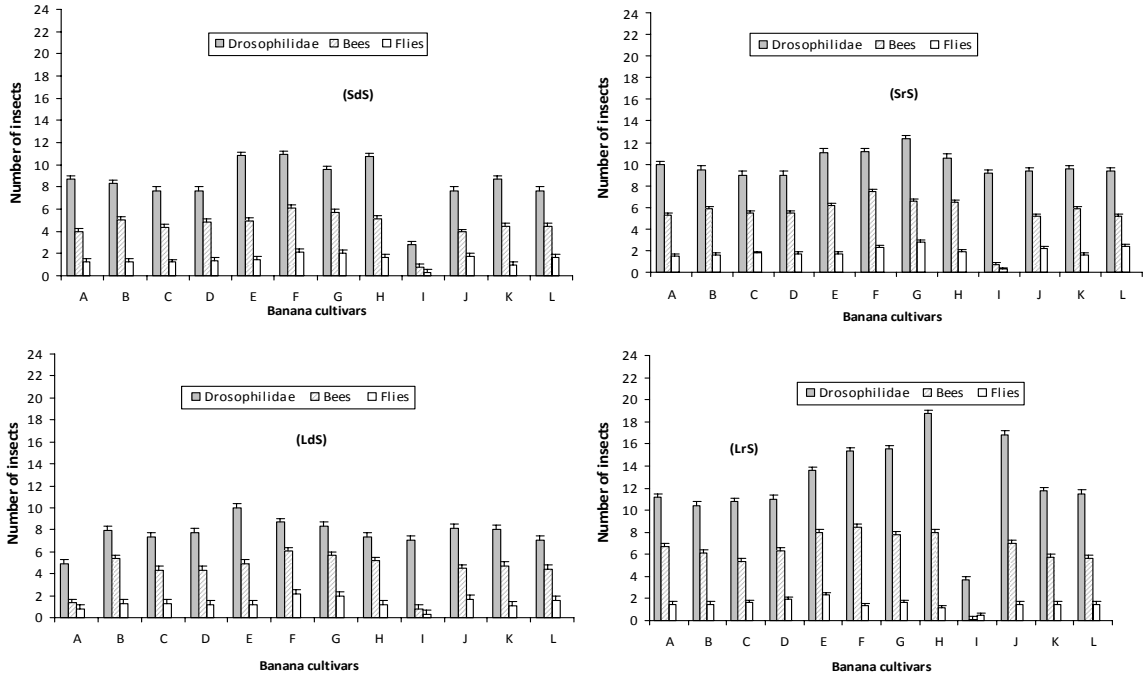

Figure 6. Prevalence of insects across banana cultivars grown in the High Altitude during the Short dry Season (SdS), Short rainy Season (SrS), Long dry Season (LdS) and the Long rainy Season (LrS). Error bars indicate the SEM. Letters at the 'X' axis stand for: (A) Kamaramasenge, (B) Gros Michel, (C) Igisukari, (D) Poyo, (E) Intuntu, (F) Ingame, (G) Kayinja, (H) Incakara, (I) Nkazikamwe, (J) Inyarwanda, (K) FHIA17, (L) FHIA25

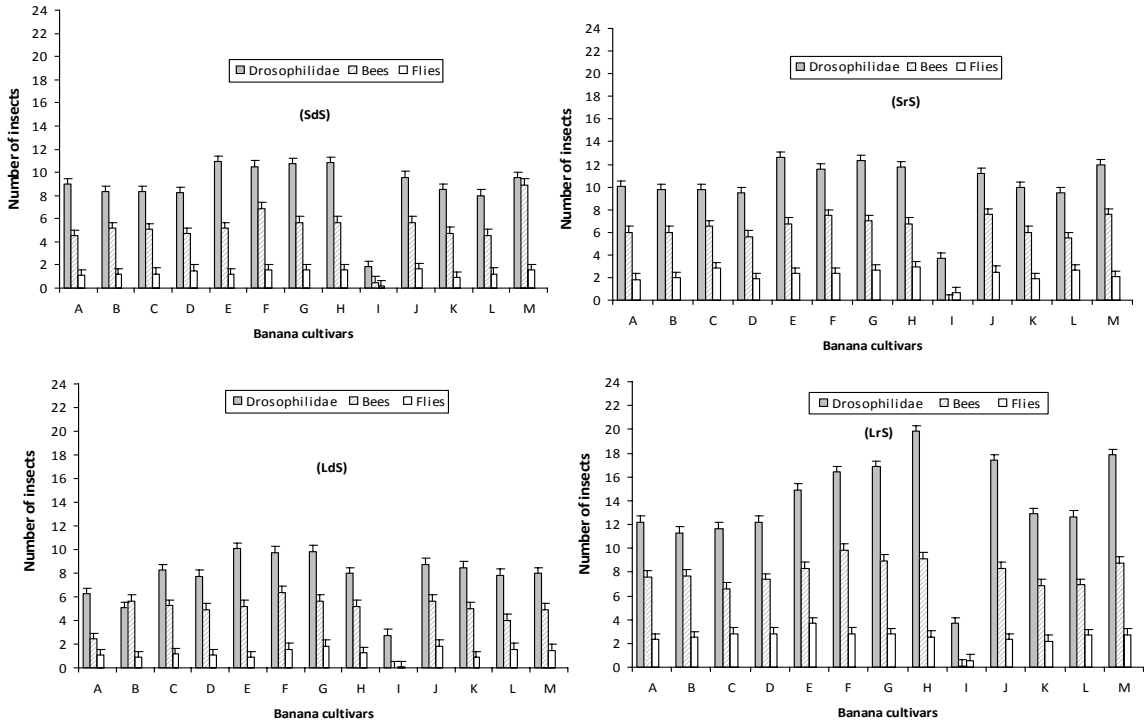

Figure 7. Prevalence of insects across banana cultivars grown in the Medium Altitude during the Short dry Season (SdS), Short rainy Season (SrS), Long dry Season (LdS) and the Long rainy Season (LrS). Error bars indicate the SEM. Letters at the 'X' axis stand for: (A) Kamaramasenge, (B) Gros Michel, (C) Igisukari, (D) Poyo, (E) Intuntu, (F) Ingame, (G) Kayinja, (H) Incakara, (I) Nkazikamwe, (J) Inyarwanda, (K) FHIA17, (L) FHIA25, (M) Nyiramabuye

As per previous scenarios (activity of insects vis- a -vis time of day and seasons of year), fruit flies, bees and other flies were the most predominant insect species across all the banana genotypes in all the study areas. The fruit flies were most attracted by beer and cooking banana cultivars, while dessert banana cultivars attracted more bees compared with other insect species (Figures 5 - 8). Higher prevalence of fruit flies was recorded on the beer banana 'Intuntu' and the cooking banana variety 'Injagi', while bees were more active on the beer cultivars 'Ingumba' 'Kayinja' 'Intuntu' and 'Nyiramabuye'. No significant differences were noted on the prevalence of flies. The activity of the three insect species was very low on the banana cultivars 'Nkazikamwe', 'Impura' and 'Ikinyangurube' (Figures 5 – 8) that have persistent floral bracts.

DISCUSSION

The present study was carried out in an attempt to understand the dynamics of insect vectors of Xanthomonas wilt of banana across four main agro-ecologies and banana cultivars grown in Rwanda. It is expected that knowledge of these aspects will help in designing improved management practices for the disease. Results showed that insect vectors of XW varied horizontally (with the four agro-ecologies) and vertically (with annual seasons and time of the day). The prevalence of insects also varied across banana cultivars grown in Rwanda. All the factors considered for the present study influenced the disease (XW) incidence as they showed significant influence on the dynamics of insect vectors of XW. According to current findings, banana cultivar distribution varied across banana growing areas. Similar results were reported from Kenya (Nguthi, 1998). A relatively higher diversity of banana cultivars was recorded in the low altitude and the Lake Kivu border region. Similar findings were also reported in two studies conducted on banana cultivar distribution in Rwanda in 2001 (Nsabimana *et al*., 2008) and in 2007 (Ocimati *et al.,* 2014).

Whereas all banana cultivars in East Africa are susceptible to XW disease (Ssekiwoko *et al*., 2006), susceptibility to floral infections was observed to vary with banana cultivars. High incidence of male bud infections were observed among beer bananas 'Kayinja' (ABB), 'Ingumba' (AAA-EA), 'Nyiramabuye' (AAA-EA) and the dessert banana cultivar 'Kamaramasenge' (AAB; elsewhere known as 'Sukari ndizi'). This study confirms the role of insects in the high XW incidence banana production systems.

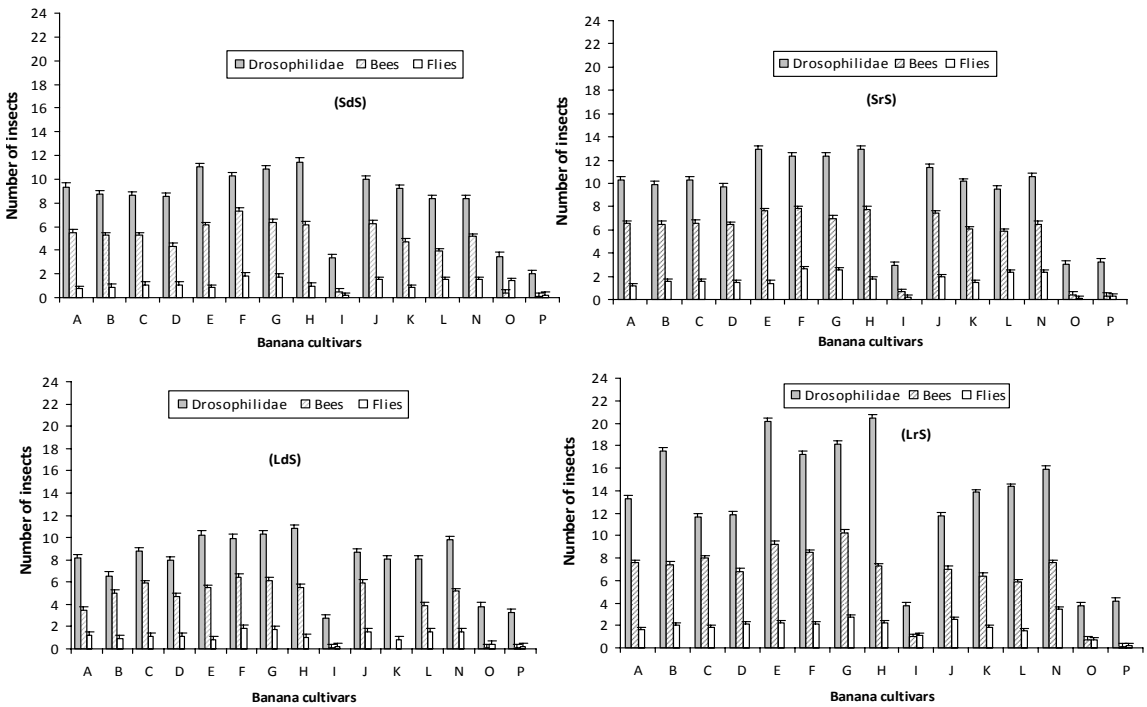

Figure 8. Prevalence of insects across banana cultivars grown in the Kivu Lake Border during the Short dry Season (SdS), Short rainy Season (SrS), Long dry Season (LdS) and the Long rainy Season (LrS). Error bars indicate the SEM. Letters at the 'X' axis stand for: (A) Kamaramasenge, (B) Gros Michel, (C) Igisukari, (D) Poyo, (E) Intuntu, (F) Ingame, (G) Kayinja, (H) Incakara, (I) Nkazikamwe, (J) Inyarwanda, (K) FHIA17, (L) FHIA25, (N) Umuzibo and (O) Impura, (P) Ikinyangurube

Higher male bud infections have been reported in the beer banana variety 'Kayinja' (*Musa* ABB) compared with a 'Dwarf Cavendish' in Ethiopia and the east African highland bananas in Uganda (Biruma *et al*., 2007;Tinzaara *et al.,* 2006; Shimelash *et al.,* 2008;). Higher incidence of insect mediated floral XW infection was also reported in 'Kayinja' dominated banana areas (Addis *et al.,* 2004). This study however, reveals cultivars other than 'Kayinja', including east African highland banana cultivars (e.g.: 'Ingumba', 'Nyiramabuye', 'Ingame') and dessert cultivar 'Kamaramasenge' to also be highly susceptible to insect mediated floral infections. The presence of these cultivars, just like 'Kayinja', in a system may also drive insect mediated infections.

Generally, the incidence of Xanthomonas wilt of banana varied significantly (P<0.001) across banana cultivars grown in Rwanda. Higher disease incidence was recorded among dessert and beer bananas. The beer banana 'Kayinja' (*Musa* ABB) and the dessert banana 'Kamaramasenge' (*Musa* AAB) showed high susceptibility to XW-male bud infection with 3.2% and 3.0%, respectively (Figure 9). These results revealed other additional cultivars similar to 'Kayinja' were susceptible to insect mediated infections. Banana cultivars 'Ikinyangurube' (AAA dessert), 'Impura' (AAA-EA) and 'Nkazikamwe' (AAA-EA) had persistent male bud bracts and neuter flowers and were thus not susceptible to XW male bud infection. Cultivars with persistent male bracts and flowers have long been reported to escape insect transmission (Biruma *et al*., 2007).

However, few 'Nkazikamwe' plants were observed to show floral symptoms, which could be attributed to tool infections. Late floral symptoms were observed in plants that were inoculated through the corm using farm tools (Ocimati *et al.,* 2013). A diversity of insects was recorded across the four study areas and annual seasons. The dynamics of insect communities (size, density, and distribution of insect populations) have been reported to vary in space and time and this is strongly influenced by environmental heterogeneity (Dobzhansky and Pavan, 1950), (Valadao *et al*., 2010 and Wolda *et al*., 1978). The most prevalent insects recorded on banana male buds were fruit flies, other flies, bees and wasps. Similar observations were noted in studies conducted in Uganda and Ethiopia (Shimelash *et al.,* 2008) and (Tinzaara *et al.,* 2006). The activity of insects generally differed with the time of the day.

Fruit flies were more active on male buds in the morning and evening. Such results were also reported in a similar study (Masanori, 1973). The crepuscular activity of these insects is a behavioral adaptation controlled by light intensity to avoid desiccation (Pavan *et al*., 1950 ; Mitchell and Epling, 1951);(. The activity of flies was similar to

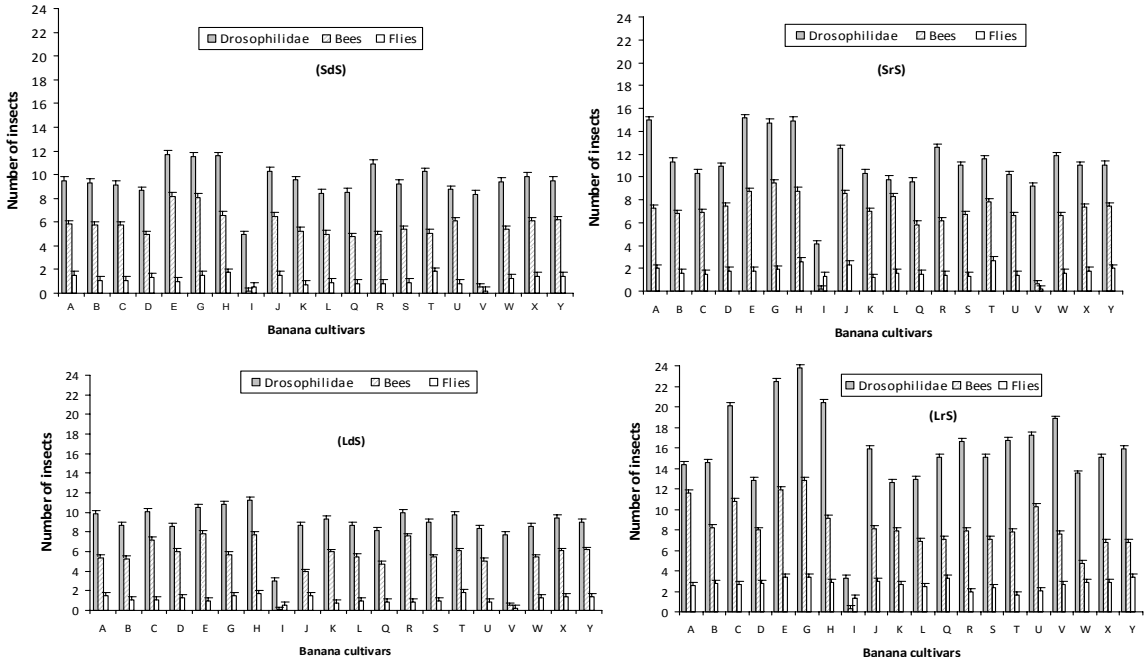

Figure 9. Prevalence of insects across banana cultivars grown in the Low Altitude during the Short dry Season (SdS), Short rainy Season (SrS), Long dry Season (LdS) and the Long rainy Season (LrS). Error bars indicate the SEM. Letters at the 'X' axis stand for: (A) Kamaramasenge, (B) Gros Michel, (C) Igisukari, (D) Poyo, (E) Intuntu, (G) Kayinja, (H) Injagi, (I) Nkazikamwe, (J) Inyarwanda, (K) FHIA17, (L) FHIA25, (P) Ingagara, (Q) Ingaju, (R) Ingenge-cooking, (S) Ingenge-beer, (T) Ingumba, (U) Intutsi, (V) Inzirabahima, (W) Inzirabushera, (X) Kibuzi

one recorded for fruit flies. Normally, fly species are reported more active on nectar plants with peaks in the morning and evening (Heatwole *et al*., 1981). Some banana cultivars have a peak of nectar production near midday and midnight but most flower opening occurs near dawn and sunset. The total resource available to pollinators will be a function of the rate of nectar production and the numbers of flowers open (Liu, 2002a). It has been also reported that the greatest amount of resource is available from 8 a.m. to 12 noon and from 10 p.m. to 2 a.m (Liu *et al.,* 2002b). This matches the timing of most visits. More bees were captured in the period between 10 am and 12 o'clock. Similar observation were made in previous studies in which, bees had unimodal peaks centering on midday (Heatwole *et al*., 1981). Wasps were more active between 10 am and 3 pm. It has been reported that the temperature is the most important factor that influences foraging of wasp species (Canevazzi *et al*., 2011). The activity of ants was consistent throughout the day. Ants in general are reported active at all hours of the day and night and time of activity peaks depend on the weather conditions (Heatwole *et al*., 1981).

Beetles are mostly active during twilight and at night or bimodally (morning and evening) except in the early rainy season when they are strongly day-active (Kenagy and Stevenson, 1982). However, no correlation was observed between beetle activity and weather patterns in the current study. Similar observations have been reported (Heatwole *et al*., 1981). This could be because beetles are strongly day-active in the early rainy season and mostly active during twilight and at night or bimodally (morning and evening) during other seasons (Kenagy and Stevenson, 1982). It was generally noted that insects were more active during the rainy season. The tendency for several insect species to be active during rainy weather especially in morning and evening may be linked to the fact that nectar resources are most heavily utilized at such time (Heatwole *et al*., 1981) Higher XW incidence was observed in the long rainy season in this study. Similar observations have been reported elsewhere (Biruma *et al*., 2007). The observed high activity (frequency and population) of insect vectors of *Xcm* during the rainy season could partially explain the high XW incidence in the rainy season. More insects were captured from the low altitude and the Lake Kivu border regions.

A higher prevalence of floral infections was observed in the low altitude and Lake Kivu border regions while no floral XW infections were recorded in the high altitude area. The high floral XW incidence in these areas correlates with the high insect activity. Fewer male bud infections were reported at altitudes above 1,700 masl compared with a lower altitude in Ethiopia (Shimelash *et al.,* 2008). The observed high activity of insect vectors of *Xcm* in the low altitude areas could partially explain the high XW incidence at the lower altitudes. In the current study, much of AAA-EA highland bananas are grown in the high altitude areas that do not support high numbers of the insect vectors, which could have also contributed to the observed low incidence of floral infections on these cultivars. Yellow colonies characteristic of *Xcm* were isolated from all the captured insect groups, with a higher frequency and load isolated from fruit flies, true flies, bees and wasps. Though ants and beetles carried the bacteria, their ability to spread it seems to be rare. These results are consistent with findings of similar studies in the DR Congo (Fiaboe *et al.,* 2008) Ethiopia (Shimelash *et al.,* 2008) and in Uganda (Tinzaara *et al.,* 2006). It should however be noted that insects such as the ants and fruit flies spend much of their life time on a few plants and may only cause localized spread of the disease.

A diversity of insect species was observed visiting banana inflorescences. The role of insects in the spread of XW disease in banana genotypes with non-persistent bracts has been demonstrated. Among the identified insect species, relatively higher populations of fruit flies, bees, other flies and wasps were observed active on male buds of banana cultivars in this study. The other insect species (i.e. ants and beetles) occurred in low numbers on banana male buds. The *Xcm* bacteria were isolated from all the insects species sampled, though ants and beetles carried low bacterial load compared with other insect species. The low number of ants and beetles carrying *Xcm* and their low activity suggest that their contribution in the spread of *Xcm* is likely to be very low. This study confirms bees, fruit flies, other flies and wasps as key insect vectors of XW disease.

Insect population varied with banana cultivars, with 'Kayinja', Nyiramabuye', Ingumba', 'Injagi' and other cultivars in the same clone set ('Incakara' and 'Barabeshya'), and 'Kamaramasenge' attracting more insects than other cultivars. Higher XW incidence has been reported in 'Kayinja' dominated areas in central Uganda. However, the distribution of this cultivar and others such as 'Kivuvu' (*Musa* ABB) and 'Gisubi' (*Musa* ABB) and 'Kamaramasenge' were sporadic. It is also eminent that several other cultivars, such as 'Ingumba' and 'Injagi' as observed in this studycan, like 'Kayinja' significantly influence XW spread. The banana cultivars 'Impura', 'Nkazikamwa' and 'Ikinyangurube' were recorded to possess persistent male buds and hence were not susceptible to transmission of *Xcm* by insect vectors. Insect vectors of *Xcm* also varied with the altitude, seasons and the time of the day. Higher insect activity was recorded in the low altitude zone in this study. Incidentally this correlated with the highest incidence of floral infections,

suggesting that disease prevalence was influenced by the high activity of the insects. Insect population and disease incidence declined with an increase in altitude.

The highest insect activity occurred during the long rainy season and least in the long dry season. This could explain the high XW incidence that has been reported in the rainy seasons. fruit flies and other flies were specifically more active during the early morning while bees and wasps were more active in the period ranging between 10 pm and 12 noon. Further evaluation of the severely affected germplasm in this study for susceptibility to insect mediated infection under controlled conditions in regions with high XW disease pressure and at a low altitude is recommended.It is also recommended that, banana cultivars with persistent male buds be promoted as they were observed to escape XW infections via insect transmission. Genetic improvement for banana cultivars such as 'Ikinyangurube' and 'Impura' should be envisaged as the two cultivars are currently not preferred by farmers due to their low yields. The lack of the yellow color at ripening in the dessert banana 'Ikinyangurube' is also blamed for its low adoption by farmers. Timely and proper de-budding (removal of the male floral bud) should contine to be advocated and emphasized during periods and in areas with high prevalence of insect vectors of *Xcm*.

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BIOPESTICIDES AND BENEFICIAL ORGANISMS IN AGRICULTURAE

БИОПЕСТИЦИДЫ И ПОЛЕЗНЫЕ ОРГАНИЗМЫ Б СЕЛЬСКОМ ХОЗЯЙСТВЕ

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ПРОИЗВОДСТВО И ПРИМЕНЕНИЕ БИОЛОГИЧЕСКИХ ПРЕПАРАТОВ ДЛЯ ЗАЩИТЫ РАСТЕНИЙ В РОССИЙСКОЙ ФЕДЕРАЦИИ

Андрей Владимирович Живых

кандидат сельскохозяйственных наук,
начальник отдела защиты растений Федерального государственного бюджетного учреждения «Российский сельскохозяйственный цент (ФГБУ «Россельхозцентр»)
av_zh@mail.ru

На современном уровне ведения агропроизводства доказано, что решить задачу борьбы с вредителями и болезнями растений только посредством массового применения химических пестицидов невозможно. Использование биологического метода защиты растений (биометода) наравне с химическим приемлемо и перспективно. Подобный подход препятствует появлению устойчивости вредителей и болезней, а также решает проблемы снижения загрязнения среды и сельскохозяйственной продукции остаточными количествами пестицидов.

Биопрепараты могут быть использованы для формирования экологически ориентированной системы защиты растений или включаться в систему интегрированной защиты, снижая пестицидную нагрузку на агроценозы. Важным достоинством биопрепаратов является и то, что их использование способствует сохранению биоразнообразия окружающей среды и благоприятствует восстановлению естественной саморегуляции биоценозов.

Введение в системы защиты растений биопрепаратов обеспечивает увеличение урожая основных культур и повышение качества сельскохозяйственной продукции, возможность отказа от использования ряда дорогостоящих пестицидов, оздоровление почвенной микробиоты, переориентацию хозяйств на производство экологически чистой продукции.

В настоящее время развитию биологического метода защиты растений в Российской Федерации способствует ряд постановлений и программ. Согласно «Комплексной программе развития биотехнологии в Российской Федерации на период до 2020 г» признается, что « ... развитие направления биологической защиты растений ведет к значительному снижению химической нагрузки на растениеводство, способствуя долгосрочной конкурентоспособности сектора».

Согласно постановлению правительства Российской Федерации №717 от 14.07.2012 г «О государственной программе развития сельского хозяйства и регулирования рынков сельскохозяйственной продукции, сырья и продовольствия на 2013 - 2020 годы» динамика развития агропромышленного комплекса до 2020 года будет формироваться под воздействием разнонаправленных факторов. В прогнозный период одной из значимых тенденций будет дальнейшая экологизация и биологизация агропромышленного производства на основе применения новых технологий в растениеводстве, животноводстве и пищевой промышленности в целях сохранения природного потенциала и повышения безопасности пищевых продуктов.

В России объем продаж биопестицидов оценивается на уровне 200 млн. руб. в год. Применяются они ежегодно на площади около 1-1,3 млн. га в открытом грунте (по данным ФГБУ «Россельхозцентр»), что составляет 1,2-1,7% от общей площади пестицидных обработок.

В мировом масштабе развитие современного уровня защиты растений трудно представить без всевозрастающей роли биологического метода. Достигнутый уровень билогизации растениеводства варьирует в отдельных странах - от 1,5-2% в Соединенных Штатах Америки до 9-10% в Швеции. В Германии и Англии на значительных площадях сельскохозяйственных угодий реализуется идея полного отказа от применения средств химической защиты растений или, по крайней мере, предоставления биометоду значительных преимуществ.

Таблица 1. Объемы производства биологических пестицидов растений в России в 2012-2014 гг (тонн)

Регион	Произведено 2012 г.		Произведено 2013 г.		Произведено весной - летом 2014 г.	
	Всего в России	в т. ч. в филиалах Россельхозцентр	Всего в России	в т. ч. в филиалах Россельхозцентр	Всего в России	в т. ч. в филиалах Россельхозцентр
Российская Федерация	981,1	681,4	1471,47	923,11	683,64	381,75
Центральный федеральный округ	70,3	67,2	170,08	164,95	76,05	71,25
Северо-Западный федеральный округ	8,8	8,8	10,25	10,25	12,03	12,03
Южный федеральный округ	345	64,2	543,76	52,09	323,61	32,78
Северо-Кавказский федеральный округ	310,4	310,4	411,21	411,21	69,10	69,10
Приволжский федеральный округ	219,1	216,3	302,4	263,86	184,63	178,37
Уральский федеральный округ	2,3	2,3	1,96	1,96	0,00	0,00
Сибирский федеральный округ	24,6	11,6	31,6	18,58	18,10	18,10
Дальневосточный федеральный округ	0,6	0,6	0,21	0,21	0,13	0,13

Одним из основных производителей биологических средств защиты растений являются лаборатории ФГБУ «Россельхозцентр». Объем произведенных биопрепаратов составляет около 55-62 % от общего объема произведенных биопрепаратов в Российской Федерации, энтомофагов – около 56-61 %. Наличие филиалов ФГБУ «Россельхозцентр» в 77 субъектах Российской Федерации дает возможность пропаганды использования биологического метода защиты растений метода и биопрепаратов по всей стране.

В ФГБУ «Россельхозцентр» в весенне-летний период 2014 г производством биопестицидов было занято 29 филиалов, энтомофагов – 4 филиала. В настоящее время в филиалах нашей организации производятся энтомофаги: трихограмма (*Trichogramma evanescens*), златоглазка (*Chrysoperla carnea*), габробракон (*Habrobracon hebetor*) ; из биопестицидов - в основном биофунгицид ризоплан (на основе *Pseudomonas fluorescens*), родентицид бактороденцид (на основе бактерий *Salmonella enteritidis var. Issatchenko*) и др., из агрохимикатов – ризоторфин (на основе бактерий из рода *Rhizobium*).

В последние годы в России отмечается рост производства биопестицидов, так в 2012 г объем производства составил 981 т, в 2013 г – 1471 т. В биолабораториях ФГБУ «Россельхозцентр» также прослеживается подобный рост производства биологических средств защиты растений, так в 2012 г произведено 681 т, в 2013 г - 923 т (табл. 1).

Наибольшие объемы производства биопестицидов в филиалах ФГБУ «Россельхозцентр» в 2013 г отмечались в Северо-Кавказском (411,2 т) и Приволжском (263,9 т) федеральных округах. Основными производителями биопрепаратов по данным 2013 г являются филиалы ФГБУ «Россельхозцентр» по Ставропольскому краю (393,9 т), Республике Татарстан (99,4 т), Кировской области (60,3 т).

Производство энтомофагов в России составляет 11-12 млрд. экз энтомофагов ежегодно. В филиалах ФГБУ «Россельхозцентр» производство энтомофагов в 2013 г. составило 7279,3 млн. экз, в 2012 г – 8079,9 млн. экз. В больших количествах от общего объема разводили трихограмму. В 2013 г. из филиалов ФГБУ «Россельхозцентр» большое количество энтомофагов наработано в Белгородском филиале 4400 млн. экз., в Ставропольском крае – 1184,8 млн. экз., в Республике Кабардино-Балкария – 1280 млн. экз., в Республике Татарстан – 1010,9 млн. экз. Кроме этого в 2013 г была произведена златоглазка (3,28 млн. экз. в Республике Татарстан) (табл. 2).

В весенне-летний период 2014 г в Российской Федерации биологические средства защиты растений и энтомофаги были применены на площади 945 тыс. га, в 2013 г. они применялись на 1304,7 тыс. га, в 2012 г – 1218 тыс. га. В весенне-летний период 2014 г в наибольшем объеме биопрепараты были применены в Северо-Кавказском федеральном округе (326,4 тыс. га), в Центральном федеральном округе (235,6 тыс. га) и в Южном федеральном округе (218,9 тыс. га).

Объемы предпосевной обработки и протравливания семенного материала в весенне-летний период 2014 г в Российской Федерации с использованием биопрепаратов на основе живых микроорганизмов был проведен в объеме 68,3 тыс. т семян и 7,3 тыс. т картофеля.

Таблица 2. Объемы производства энтомофагов в России в 2012-2014 гг (млн. шт)

Регион	Произведено 2012 г.		Произведено 2013 г.		Произведено весной - летом 2014 г.	
	Всего в России	в т. ч. в филиалах Россельхоз-центр	Всего в России	в т. ч. в филиалах Россельхоз-центр	Всего в России	в т. ч. в филиалах Россельхоз-центр
Российская Федерация	14425,8	8079,9	12094,9	7279,3	11996,17	7372,91
Центральный федеральный округ	6497,5	4040	5462,6	4400	5497,15	4292,00
Северо-Западный федеральный округ	252,6	0	215,1	0	229,56	0,00
Южный федеральный округ	975,4	0	633,3	0	555,18	0,00
Северо-Кавказский федеральный округ	3022,8	3022,8	2464,8	2464,7	2925,70	2925,70
Приволжский федеральный округ	1799,6	1017,1	1479,6	414,6	1142,44	155,21
Уральский федеральный округ	26,7	0	27,6	0	27,60	0,00
Сибирский федеральный округ	1851,2	0	1811,9	0	1618,54	0,00
Дальневосточный федеральный округ	0	0	0	0	0,00	0,00

Важной задачей на ближайшее время в России останется расширение количества биопрепаратов в «Списке пестицидов и агрохимикатов, разрешенных к применению на территории Российской Федерации». Биопрепараты, зарегистрированные и включенные в «Список», требуют дальнейших испытаний для увеличения спектра их применения на большее количество сельскохозяйственных культур.

Основными потребителями биологических средств защиты растений, производимых в России являются крупные сельскохозяйственные предприятия, агрохолдинги и фермерские хозяйства. Они располагают большой площадью сельхозугодий и потенциально образуют наиболее ёмкую часть потребительского рынка. Тепличные хозяйства в силу их деятельности и экологичности биопрепаратов также заинтересованы в развитии применения биометода на своих площадях.

В ближайшие годы в биолабораториях ФГБУ «Россельхозцентр» особое внимание будет уделено увеличению производительности и эффективности работы биолабораторий, расширению ассортимента выпускаемой продукции, улучшению качества биопрепаратов и укреплению позиций биолабораторий ФГБУ «Россельхозцентр» на российском рынке биопестицидов.

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PERSPECTIVES OF BIOLOGICAL CONTROL TO THE SOUTH AMERICAN TOMATO MOTH, *Tuta absoluta* IN GEORGIA

Manana Kakhadze, Tsisia Chkhubianishvili, Iatamze Malania, Mariam Chubinishvili, Rusudan Skhirtladze, Irine Rijamadze and Nino Nazarashvili

NLE Agricultural University of Georgia, Kanchaveli Institute of Plant Protection, Biocontrol laboratory, 240, Agmashenebeli Alley, Tbilisi 0591, Georgia
manana.kakhadze@gmail.com

ABSTRACT

The South American tomato moth, *Tuta absoluta* (Povolny) (Lepidoptera*:* Gelechiidae) a new, invasive pest, introduced from Turkey from 2011 revealed in tomato seedlings in the Western Georgia (Khobi, village Khorga). Cultivated and wild plants from *Solonaceae* family are the hosts for pest, but the tomato, eggplant and pepper considered as the most important. In recent years the rapid growth of *T. absoluta* population has caused the significant damage of tomato plants in greenhouses. The yield loss of tomatoes exceed 35-50% and possible to reach 60-80%. During vegetation season *T. absoluta* can develop 10-12 generations and consequently necessery to conduct control measures. The usage of environmentally safe means, especially in greenhouses, is advisable. Regarding this, study was conducted on the action of bacterial preparation „*Delphin*" (on the base *Bacillus thuringiensis* subsp. Kurstaki, strain SA-11) and the entomopathogenic nematode - *Steinerma feltiae*, "Georgian strain". 15 tomato plants were settled with different instars of *T. absoluta* 8-12 larvae and treated by „Delphin" 1% solution in laboratory. Nematode suspension (500 IJs/ml) was used for 8-12 larvae treatment and tomato pots were placed at 23-25°C and 60-70% RH. The tomato test plants were treated twice by bacterial and nematode preparations to 7 days interval, whereas control was applied with distilled water. The mortality of larvae was compared to the control, using Abbott's formula. The action of biological means for 3, 5, 7, 10 days after treatment were detected. Larvae infestation was detected after 48 and 72 hr. Data was analysed by two-way ANOVA (p=0.05). The mortality index for *"Delphin"* preparation achieved to 95.1%, for nematode suspension – 79.2%. According to data of preliminary laboratory assays, the research will be continued to establish biological effectiveness in greenhouse conditions.

Key words: Tomato moth, IPM, Bacterial preparation, Entomopathogenic nematode

INTRODUCTION

Besides to intensive development of greenhouse farms (more than 400-500 ha) the number of introduced pests have increased in Georgia during last years. According to Ministry of Agriculture of Georgia 2011 year's observation data (internet sources), the South American tomato moth, *Tuta absoluta* (Povolny) (Lepidoptera: Gelechiidae) and the serpentine leaf miner, *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae), as new invasive pests have been added to the greenhouse pests' list. Our observations show, that among other pests: the greenhouse whitefly - *Trialeurodes vaporariorum* (Westw.), the melon aphid - Aphis *gossypi* (Glow.), the green peach aphid - *Myzodes persicae* (Sulz), the red spider mite - *Tetranychus urticae* (L.), *L. trifolii* - thripses (Chubinishvili *et al.,* 2013), *T. absoluta* is more economic pest of tomato in greenhouses of Georgia, where the chemical pesticides treatment can't give satisfaction results.

Tuta absoluta generations	April			May			June			July			August			September			October			November March		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
I	· 0 ■	· 0 ■	· 0 ■																					
II																								
III																								
IV																								
V																								
VI																								
VII																								
VIII																								
VIII																								
IX																								
X																								
XI																								
XII																					· 0 ■	· 0 ■	· 0 ■	· 0 ■

Optional Symbols: · Egg; 0 Pupa; ▬ Durations of one generation; ■ Overwintering imago.

Figure 1. South American tomato moth, *Tuta absoluta* Povolny (*Lepidoptera: Gelechiidae)* phenological calendar in Mtskheta (Eastern Georgia)

T. absoluta is considered as an economically damaging pest in many countries. It is distributed from South American Continent (Garcia,1982), throughout many European countries (EPPO, 2005). In Europe it was initially detected in the Iberian Peninsula in 2006 (Urbaneja *et al.,* 2007). Since it has rapidly moved across the Mediterranean area and has been detected in France, Italy and the United Kingdom (UK) (Desneux *et al*., 2010; Urbaneja *et al.,* 2012). Initially the struggle against pest was started by chemical treatments, hence soon these methods were reduced because of rapid resistance development of the pest (Cabello *et al*. 2012). Among all control measures to *T.absoluta,* the biological means had priority in integrated pest management (IPM) system.

MATERIAL AND METHODS

T. absoluta damage and the average density of pest population were calculated by point system by using of accepted method (Dospekhov, 1979). The infested tomato leaves were collected in greenhouses and transfered to laboratory.The preliminary laboratory experiments were carried out on action of bacterial preparation „*Delphin*" (based on *Bacillus thuringiensis* subsp. Kurstaki, strain SA-11) and the entomopathogenic nematode (EPN) - *Steinerma feltiae*, "Georgian strain". The different instars larvae (8-10) were settled on 15 tomato potted plants and treated with 1% of „Delphin" solution. Nematode suspension (500 IJs/ml) was used for treatment 15 tomato seedlings, settled with 8-12 larvae each at 23-25°C and 60- 70% RH conditions. In both cases (bacteria and nematode) the tomato plants were treated twice of 7 days interval and control variants applied with distilled water. The mortality of tested cadavers was compared to control mortality using Abbott's formula (Abbott, 1925). The action of biological means for 3, 5, 7, 10 days after treatment were detected. The infected larvae were observed after 48 - 72 hr on 10th day of experiment. Data was analysed by two – way ANOVA (p=0.05).

RESULTS AND DISCUSSION

Since 2011 *T. absoluta* is limited spread quarantine pest in Georgia according to data from the Ministry of Agriculture of Georgia. Initially the pest revealed in tomato seedlings imported from Turkey in the Western Georgia, then it was rapidly spread the Eastern regions of country, in suburbs of Tbilisi, Mtskheta, Marneuli, Gardabani greenhouses and other private farms, were the tomato Holland sort "Big-Bef" was distributed.

The phenological calendar of pest insect, *T. absoluta* was compiled for Mtskheta region (Figure, 1). The great damage on leaves was achieved to 50-75% (Figure 2).

Figure 2. Damaged tomato leaves and fruits with *T. absoluta* in Mtskheta

The action of biological means after treatment is presented (Figure 3, 4). Two-way analysis were used to compare *T.absoluta* corrected mortality to biological means *"Delfin"* and "*Georgian strain"* and their interaction for different days (p value = 0.05) (Figure 5).

The mortality index of first and second instars of *T.absoluta* larvae, treated by Delfine 1%, has achieved to 95.1% after 5-10 days.

In the case of EPN "Georgian strain" 500 IJs/ml, it was established that larvae invasion has achieved 79.2%. The high volume of EPN, *Steinernema feltiae* foliar sprays is described by Jacobson & Martin (2011). Considering the results, Delphin and EPN can be included to *T. absoluta* IPM system. It should be noted that third and fourth instars larvae are protected by leaf folds or fruits and their control was difficult (Poe, 1973). Insect populations may be controlled at the first or second larval instars with recommended bio-insecticides.

Figure 3. *T.absoluta* Mortality in laboratory

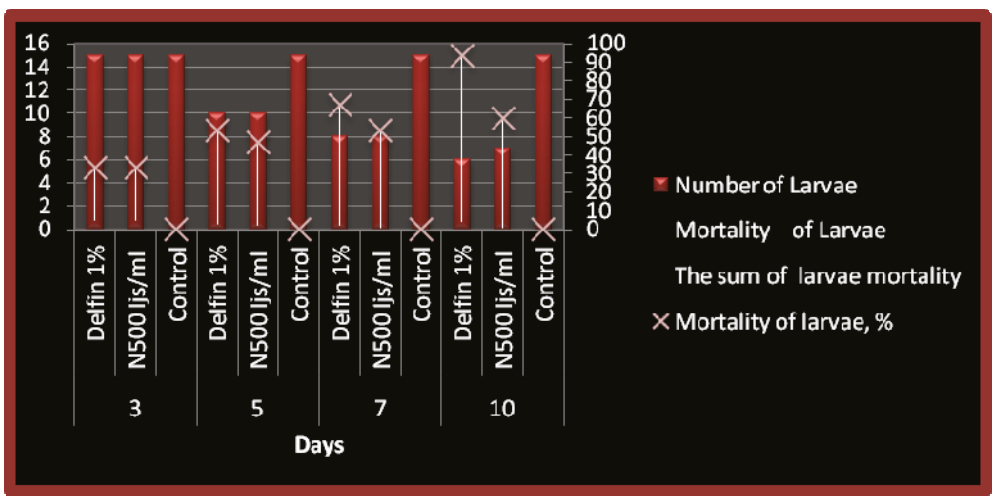

Figure 4. The corrected mortality of *T.absoluta* larvae for different days

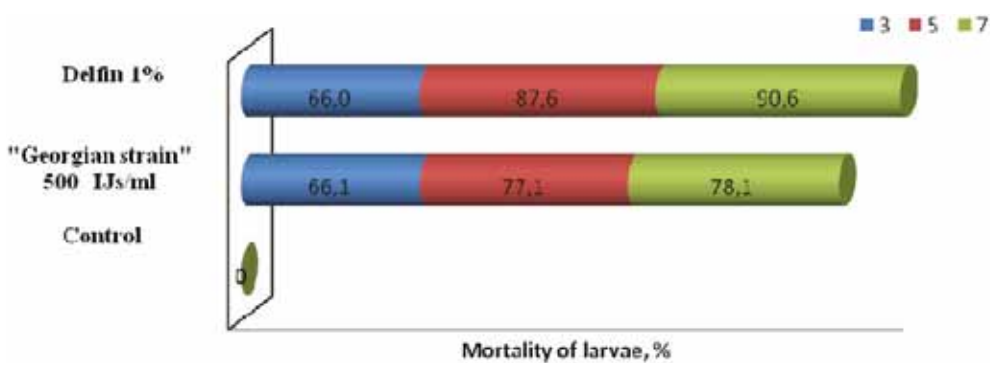

Figure 5. Interaction of biological means "*Delfin"* and "*Georgian strain"* to *T.absoluta*

This study is first attempt to control the South American tomato moth, *Tuta absoluta* with biological means in Georgia. Based on the results obtained in preliminary laboratory experiments the investigations will be continue to establishment of biological effectiveness in greenhouse conditions. The tested safe biological means will take an important place for vegetables protection from pest organisms in the system of integrated pest management (IPM).

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PLANT GROWTH PROMOTING RHIZOBACTERIA AS A POSSIBLE PART OF IWM

Dragana Božić[1], Danijela Pavlović[2], Marija Sarić-Krsmanović[3] and Sava Vrbničanin[1]

[1]University of Belgrade, Faculty of Agriculture, Nemanjina 6, Belgrade-Serbia
[2]Institute for Plant Protection and Environment, Teodora Drajzera 9, Belgrade, Serbia
[3]Institute of Pesticides and Environmental Protection, Banatska 31b, Belgrade, Serbia
sava@agrif.bg.ac.rs

ABSTRACT

The effects of different PGPR including *Azotobacter chroococcum, Bacillus amyloliquefaciens, B. circulans, B. licheniformis, B.megatherium, B. pumilus, B.subtilis* and *Pseudomonas fluorescens* on seed germination of several weed species (*Abuthilon theophrasti* Medik., *Amaranthus retroflexus* L., *Ambrosia artemisiifolia* L., *Datura stramonium* L., *Iva xanthifolia* Nutt., *Onopordon acanthium* L., *Sorghum halepense* (L.) Pers., *Verbascum thapsus* L.) have been tested. Seeds of each species were germinated in solutions containing the chosen bacteria in Petri dishes. Control (seed germination in water) was also included for each weed species. Germination tests were conducted in an incubator set to 25°C, in the dark. Seeds were considered to be germinated with the emergence of the radicle. Germinated seeds were counted and the percentage of germination was calculated after 8 days.

The effects of PGPR on seed germination of eight weed species were diverse depending on the combination of bacterial and weed species. As all seeds of *V.thapsus* were germinated in all treatments (different bacterial solutions and control), it was not posible to analyze the bacterial effect on the germination of this species.

The Obtained results have shown that the bacteria tested had either a stimulating or an inhibiting potential on the seed germination. These findings indicate that PGPR can be a potential tool for weed control. Therefore, future perspective is to implement the acquired knowledge in the concept of IWM.

Key words: weed, plant growth-promoting rhizobacteria (PGPR), seed germination.

INTRODUCTION

Modern weed control is oriented on using low inputs of herbicides and alternative non-chemical methods due to the decrease in the number of selective herbicides available for chemical weed control in the past decade. Also, intensive chemical control leads to the development of herbicide resistant weed populations, pollution in water and soil and residues in produces. Public concern about the detrimental effects of pesticides including herbicides on the environment is increasing. This suggests the need to search for new techniques/tools for weed control which may be environmentally friendly, easy to use and inexpensive.

Beneficial free-living soil bacteria which are usually refered to as PGPR (plant growth promoting rhizobacteria) belong to the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillium*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Serratia* (Rodriguez and Fraga, 1999; Sturz and Nowak, 2000; Sudhakar et al., 2000). They can modulate plant growth in different ways: 1) by enhancing the availability of nutrients (inducing metabolic activities by phytohormones and analogs), 2) by shifting the promotional balance, 3) by inducing defense programs such as systemic acquired resistance and induced systemic resistance, or 4) by reducing phytotoxic microbial

associations (Ping and Boland, 2004). Additionaly, Ryu et al. (2003) demonstrated that bacterial volatiles from *Bacillus subtilis* and *B. amyloliquefaciens* can promote the growth of *Arabidopsis thaliana* seedlings.

Generally, PGPR are used as inoculants for biocontrol, biofertilization and phytostimulation (Ping and Boland, 2004). Also, they can be included in integrated weed management (IWM) in two possible ways, by inhibiting or by stimulating the seed germination. Namely, if they inhibit germination of weed seeds they can reduce weediness directly and contribute to a seed bank depletion. They can attract the seeds by: 1) chemotaxis, 2) rapid colonization of the spermosphere and 3) production of enzymes and/or phytotoxins which can kill seeds prior to germination (Kremer, 1993). But on the contrary, some of them can stimulate seed germination. In that case, they can encourage uniform weed emergence, after which they could be killed in the next step of weed control. Many studies have confirmed that PGPR can promote germination and growth of different crops (Mayak et al., 1999; Egamberdiyeva, 2007), but fewer have been concerned with their effects on the germination of seeds and very young weed seedlings(Daigle et al., 2002; Ryu et al., 2003).

The general objective of this research was to explore the possibility of using PGPR as a part of IWM. But, the first step in that process is investigation of basic ecological interactions between different PGPR and seed germination of different weed species.

MATERIAL AND METHODS

The effects of some PGPR including *Azotobacter chroococcum, Bacillus amyloliquefaciens, B. circulans, B. licheniformis*, *B.megatherium*, *B. pumilus, B.subtilis* and *Pseudomonas fluorescens* on seed germination of eight weed species (*Abuthilon theophrasti* Medik., *Amaranthus retroflexus* L., *Ambrosia artemisiifolia* L., *Datura stramonium* L., *Iva xanthifolia* Nutt., *Onopordon acanthium* L., *Sorghum halepense* (L.) Pers., *Verbascum thapsus* L.) have been tested.

All bacterial strains used in the study were isolated from different rhizospheres: 1) *A. chroococcum* and *P. fluorescens* were isolated from the rhizosphere of wheat; 2) *B. megatherium* and *B. circulans* were isolated from the rhizosphere of corn; 3) *B. licheniformis, B. pumilus* and *B. amyloliquefaciens were* isolated from the manure and 5) *B. subtilis* was isolated from the compost. Incubation of weed seeds was done with 24h old inocula with cell concentration of 10^8 ml^{-1}.

Seeds of weed species were sterilized with 1% (v/v) sodium hypochlorite solution for 10 minutes. After that, seeds were rinsed with distilled water three times. Seeds of each species were germinated in 5 ml of solutions containing the chosen bacteria (Table 1) in Petri dishes. Control (seed germination in water) was also included for each weed species. Germination tests were conducted in an incubator Binder CE set to 25°C, in the dark. Seeds were considered to be germinated with the emergence of the radicle. Germinated seeds were counted and percentage of germination was calculated after 8 days. Three dishes were used for each treatment and control. Each experiment was conducted three times.

The obtained results were processed using software Statistica 5.0 by the descriptive statistics and LSD test.

Table 1. Weed species and PGPR included in study

Weed spesies	PGPR applied to selected weed species
A. artemisiifolia	*A. chroococcum*; *B. amyloliquefaciens* *B. licheniformis*; *B. pumilus*; *P. fluorescens*
A. retroflexus	*A. chroococcum*; *B. circulans*
A. theophrasti	*B. licheniformis*; *B. subtilis*; *B. megatherium*
D. stramonium	*B. licheniformis*; *B. subtilis*; *B. megatherium*
I. xanthifolia	*A. chroococcum*; *B. circulans*
O. acanthium	*B. licheniformis*; *B. subtilis*; *B. megatherium*
S. halepense	*A. chroococcum*; *B. circulans*
V. thapsus	*B. licheniformis*; *B. subtilis*; *B. megatherium*

RESULTS

The effects of PGPR on seed germination of eight weed species were diverse depending on PGPR and weed species in that order (Table 2). Generally, the highest germination was recoreded for *V. thapsus*, which obtained 100% germination in control and bacterial solutions. Among other seven species the highest germination was recorded for *A. retroflexus* (45-82%), while for all other species the percentage of seed germination after 8 days of incubation was below 45.00% and the lowest germination was recorded for *A. artemisiifolia* (0.00-7.50%).

The germination of *A. artemisiifolia* in the presence of five PGPR was different. Namely, seed germination in the *A. chroococcum* (5.94%), *B. amyloliquefaciens* (6.25%) and *B. pumilus* (7.50%) solutions was slightly higher than in control (5.63%), in *B. licheniformis* (5.07%) solution was slightly lower than in control, while *P. fluorescens* completely stopped the germination. The effects of

mentioned bacteria on *A. artemisiifolia* germination was statistically significant (P<0.05) only for *P. fluorescens*.

Although *A. retroflexus* generally had high germination, percentage of germinated seeds in control (45.00%) was significantly (P<0.05%) lower than in solutions of *A. chroococcum* (81.67%) and *B. circulans* (66.67%). Similar results were obtained for *I. xanthifolia*, which germinated better in bacterial solutions (*A. chroococcum*-18.33%; *B. circulans*- 25.00%) than in control (11.67%). The same effect of *A. chroococcum* (35.00%) on *S. halepense* was recoreded, while its germination in *B. circulans* solution was the same as in control (21.67%).

The germination of *A. theophrasti* in the presence of *B. subtilis* (28.33%) was very significantly (P<0.01) lower than in the control (43.33%). On the other hand, germination in *B. megatherium* solution was the same as in the control and in *B. licheniformis* solution slightly lower (41.67%). The effect of bacterial solutions on the germination of *D. stramonium* seeds was statistically very significant (P<0.01), in B. *licheniformis* solution the seed germination was reduced, while *B. subtilis* and *B. megatherium* solutions stimulated the seed germination. The germination of *O. acanthium* in all three bacterial solution was very significantly higher (*B. licheniformis*- 13.335; *B. subtilis*-13.335; *B. megatherium*-11.67%) than in control (6.67%).

Table 2. Effect of PGPR on seed germination of *A. theophrasti*, *A. retroflexus*, *A. artemisiifolia*, *D. stramonium*, *I. xanthifolia*, *O. acanthium*, *S. halepense* and *V. thapsus*

Species	PGPR	Germination (%)	Control: PGPR (LSD-test)
A. artemisiifolia	Control	5.63±2.81	
	A. chroococcum	5.94± 2.07	N.S.
	B. amyloliquefaciens	6.25±4.49	N.S.
	B. licheniformis	5.07±4.63	N.S.
	B. pumilus	7.50±6.12	N.S.
	P. fluorescens	0.00±0.00	*
A. retroflexus	Control	45.00±22.91	
	A. chroococcum	81.67±2.89	*
	B. circulans	66.67±30.55	*
A. theophrasti	Control	43.33±21.00	
	B. licheniformis	41.67±11.00	N.S.
	B. subtilis	28.33±5.00	**
	B. megatherium	43.33±21.00	N.S.
D. stramonium	Control	10.00±2.04	
	B. licheniformis	5.00±2.98	**
	B. subtilis	13.33±3.66	**
	B. megatherium	11.67±3.21	**
I. xanthifolia	Control	11.67±11.54	
	A. chroococcum	18.33±12.58	**
	B. circulans	25.00±10.00	**
O. acanthium	Control	6.67±3.50	
	B. licheniformis	13.33±1.09	**
	B. subtilis	13.33±7.14	**
	B. megatherium	11.67±3.67	**
S. halepense	Control	21.67±2.89	
	A. chroococcum	35.00±0.00	*
	B. circulans	21.67±2.89	N.S.
V. thapsus	Control	100.00±0.00	
	B. licheniformis	100.00±0.00	N.S.
	B. subtilis	100.00±0.00	N.S.
	B. megatherium	100.00±0.00	N.S.

**- P<0.01; *- P<0.05; N.S. - P>0.05

DISCUSSION

This paper provides an overview of the effects of the selected PGPR bacteria on seed germination of chosen weed species. As all seeds of *V. thapsus* were germinated in all treatments including the bacterial solutions and control, it was not possible to analyze bacterial effects on the germination of this species. Different bacterial solutions had diverse effects (stimulative or inhibitory) on seed germination of other seven weed species, depending on the bacterial and weed species, in that order. For some PGPR and weed species that effect was positive and for some negative (Table 2). For example, *B. licheniformis* inhibited *A. theophrasti* and *D. stramonium* seeds germination, while oposite results were obtained for *O. acanthium*. Also, *B. circulans* promoted the germination of seeds of *A. retoflexus* and *I. xanthiflolia*, while there was no effect on the germination of *S. halepense*. The stimulative effects of *Bacillus* on seed germination and plant growth were reported by many researchers (Ryu et al., 2003 ; Ahmad et al., 2006) who explained such effects as a result of the production of plant growth–promoting substances like gibberelins, indoleacetic acid, ammonia, hydrogen cyanide etc. Contrary to that, Saric and Bozic (2009) found *Bacillus* species to have inhibitory effects on the germination of *Cuscuta campestris* Yunck.

A. chroococcum has the ability to fix atmospheric N in association with plant roots and to produce plant growth-promoting supstances (Martinez-Toledo et al., 1988; Revillas et al., 2000). Due to that it has been used as a seed inoculant. In our study the solution of this bacteria promoted the germination of seeds of *A.retoflexus*, *I. xanthiflolia* and *S. halepense*, there was no effect on the germination of *A. artemisifolia*. Vrbnicanin et al. (2008) found that the mix of *A. chroococcum* and humates had a better effect on germination of *I. xanthiflolia* than pure solution of the same species.

P. fluorescens completely inhibited the germination of *A. artemisiifolia*, which is contrary to the results of plant growth promotional effects of this species obtained by Ahmad et al. (2006). Thsese results are consistent with different classifications of this bacteria. Namely, some authors have classified this bacteria as PGPR (Jaleel et al., 2007), others have classified it as a deleterious rhizobacteria (Zdor et al., 2005). But, Carrillo-Castaneda et al. (2002) concluded that the effect of this bacterial species on alfalfa seed germination depend on the conditions in which the bacterial cultures develop.

As we studied the effects of selected PGPR on different weed species in Petri dishes and as different conditions in the soil (pH, microelements, salinity) can influence the excretion of plant growth-promoting supstances (Narula and Gupta, 1986, Egamberdiyeva 2007), it may not be possible to extrapolate the results of these *in vitro* studies to soil or rhizosphere conditions. Therefore, it is necessary to continuated the studies of the effects of bacterial species in the field and based on that evaluate the possibility for their use as a part of IWM.

In summary, the results of this study indicated that the same bacteria can have opposite effects (stimulative or inhibitory) on different weed species. So, it is necessary to do screening for the effects of those bacteria on many weed species and based on that evaluate the possibility of their use in weed management. Interesting future perspectives might be to see how we can use the knowledge obtained about PGPR-seed germination interaction in integrated weed management systems.

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EFFECT OF COMPOSTING ON WEED SEEDS SURVIVAL

Dragana Božić[1], Vladimir Filipović[2], Ana Matković[1], Tatjana Marković[2] and Sava Vrbničanin[1]

[1]University of Belgrade, Faculty of Agriculture, Nemanjina 6, Belgrade-Serbia
[2] Institute of Medicinal Plant Research "Dr Josif Pančić", Belgrade, Serbia
sava@agrif.bg.ac.rs

ABSTRACT

Biological waste used for the preparation of compost can be a source of weed seeds. Survival of these seeds during composting depends on several factors, especially on temperature. Also, the method of composting and the addition of activators can have an important role for the reduction of weed seeds. Therefore, it is necessary to choose the method of composting which is the most effective in destroying the viability of weed seeds.

Compost was prepared using biological waste produced at fields of the Institute for Medicinal Plant Research "*Dr Josif Pančić*" from Belgrade, located in Pančevo. The experiment was designed with four treatments: COMP1 (control variant- biological waste without activators), COMP2 (biological waste treated with microbiological fertilizer Bactofil B-10 in the amount of 1.5 L per 10 T), COMP3 (biological waste treated with the organic NPK fertilizer Ekovital in the amount of 2 L per 10 T) and COMP4 (biological waste treated with Urea in the amount of 50 kg per 10 T). Effects of activators on weed seed survival were analyzed based on weed emergence from compost in June 2014.

Generally, the addition of different activators affected the survival of weed seeds. The highest number of emerged weed plants was recorded in COMP1, followed by COMP2 and COMP3, while the lowest number of emerged plants was obtained in COMP4. Number of weed species whose seeds survived in COMP1, COMP2 and COMP3 was 17, while in COMP4 seeds of only 8 species survived. Seeds of those 8 weed species (*Amaranthus retroflexus* L., *Artemisia vulgaris* L., *Chenopodium album* L., *Digitaria sanguinalis* L. (Scop.), *Panicum crus-galli* (L.) P. Beauv., *Portulaca oleracea* L., *Setaria viridis* (L.) P. Beauv., *Solanum nigrum* L.) survived in all treatments.

Key words: compost, seed, survival, weed

INTRODUCTION

Compost can be used as a highly valuable organic fertilizer, improver of soil structure, as well as raw material for the production of various substrates and mulches etc. It is well known that compost has many positive effects on agricultural production and environment. Namely, mature compost can serve as a sources of substances that stimulate plant growth (Chen and Aviad, 1990; Valdrighi et al., 1996). Additionally, compost can alter soil thermal regimes (Jacobowitz and Steenhuis, 1984) and increase soil moisture content (Serra-Wittling et al., 1996). It can also increase soil microbial biomass and activity (Fraser et al., 1988).

Although many benefits can be attributed to the use of compost, its use in agriculture can lead to occurrences of new problems for the farming system. Because weed and crop species can differ in their growth responses to soil conditions, application of compost might alter the balance of weed-crop interactions. Also, many materials used for making the compost are contaminated with viable weed seeds and plant pathogens. Even if material for composting does not contain weed seeds, these seeds may contaminate the compost in open-air pits

via dissemination and deposition by wind. This is the reason for concern about the presence of weed seeds in compost, which limits the use of composted organic waste in agriculture and horticulture.

In the process of production and processing of medicinal, aromatic and spice plants huge amounts of biological waste are produced. In the past period, on the territory of our country, this waste was inadequately treated, which resulted in a loss of about 680,000€ per year (Filipović and Ugrenović, 2013). The European Union Landfill Directive, which prohibits the disposal of biodegradable waste in landfills (The Council of the European Union, 1999) encourages composting and other methods of treatment of biodegradable waste as a very convenient way to reduce the amount of this kind of waste. But, compost preparation from biological waste obtained during the production and processing of medical plants is associated with the risk of contamination with viable weed seeds. Although there is a common perception that composting kills weed seeds, different factors, including interaction between weed species, temperature, time and moisture (Shiralipour and Mcconnell, 1991; Eghball and Lesoing, 2000; Larney and Blackshaw, 2003; Dahlquist et al., 2007) can affect the survival of seeds. According to our knowledge, there is no data about the effects of activators like microbiological or mineral fertilizers onthe survival of weed seeds during composting. Therefore, the aim of this study was to examine weed seeds survival during composting depending on the addition of composting activators.

MATERIAL AND METHODS

The study was performed at the experimental field of the Institute for Medicinal Plant Research "Dr Josif Pančić" from Belgrade, located in Pančevo, during 2013 and 2014. Compost was prepared using biological waste during November 2013. Biological waste contained about 78% of waste produced during primary processing of medical plants, about 18% of waste produced during extraction and distillation of different parts of medical plants and about 4% of waste produced in conventional agricultural production. Experiment was designed with four treatments including control variant COMP1 and three variants to which different activators were added: COMP2 (biological waste treated with microbiological fertilizer Bactofil B-10 in the amount of 1.5L per 10T), COMP3 (biological waste treated with organic NPK fertilizer Ekovital in the amount of 2L per 10T) and COMP4 (biological waste treated with Urea in the amount of 50 kg per 10T). Mean monthly temperatures and precipitation are summarized in Table 1. Total precipitation during composting (from November 2013 to June 2014) was 493.8mm, which may be considered as a water input to the compost pit. Also, all treatments were watered several times and one mechanical mixing was done. The effects of composting on weed seeds survival were analyzed based on the number of emerged weed plants from compost in June 2014. In all compost pits (surface area 12 m^2) emerged weed species and the number of emerged plants per pit were determined.

Table 1. Mean monthly temperatures and precipitation during the composting period at the experimental site.

Month	Mean monthly temperature (°C)	Precipitation (mm)
November	9.1	49.0
December	2.1	7.3
January	3.7	26.8
February	7.9	19.1
March	10.0	50.4
April	13.5	68.9
May	16.4	220.2
June	21.5	52.1

RESULTS

Generally, the survival of weed seeds during composting depended on the treatment. The total number of species found in all compost pits was 28 (Table 2). The highest number of emerged weed plants was recorded in COMP1 (30 plants per pit), followed by COMP2 (27 plants per pit) and COMP3 (21 plants per pit), while the lowest number of emerged plants was obtained in COMP4 (8 plants per pit). The number of weed species whose seeds survived in COMP1, COMP2 and COMP3 was 17, while in COMP4 seeds of only 8 species survived. Seeds of those 8 weed species (*Amaranthus retroflexus* L., *Artemisia vulgaris* L., *Chenopodium album* L., *Digitaria sanguinalis* L. (Scop.), *Echinochloa crus-galli* (L.) P. Beauv., *Portulaca oleracea* L., *Setaria viridis* (L.) P. Beauv., *Solanum nigrum* L.) were survived in all pits. Four species (*Achillea millefolium* L., *Bifora radians* M.B., *Convolvulus arvensis* L., *Stachys annua* L.) were found only in control treatment, while some species (*Amaranthus blitoides* S. Watson., *Anethum graveolens L., Ballota nigra* L., *Matricaria chamomilla* L., *Cuscuta sp.*, *Hibiscus trionum* L., *Linum usitatissimum* L., *Rumex crispus* L., *Silybum marianum* L. Gaertn., *Sinapis arvensis* L., *Xanthium strumarium* L.) were not present in the control treatment, but were present in the treatments with activators.

Table 2. Weed species in compost pits.

Weed species	Number of plants per pit			
	COMP1	COMP2	COMP3	COMP4
Achillea millefolium L.	1	-	-	-
Amaranthus blitoides S. Watson.	-	1	-	-
Amaranthus retroflexus L.	4	2	2	1
Anethum graveolens L.	-	1	-	-
Artemisia vulgaris L.	2	2	1	1
Ballota nigra L.	-	1	1	-
Bifora radians M.B.	1	-	-	-
Matricaria chamomilla L.*	-	1	1	-
Chenopodium album L.	2	3	2	1
Convolvulus arvensis L.	1	-	-	-
Cuscuta sp.	-	1	1	-
Digitaria sanguinalis (L.) Scop.	1	1	1	1
Hibiscus trionum L.	-	-	1	-
Lactuca serriola L.	1	-	1	-
Linum usitatissimum L.*	-	-	1	-
Echinochloa crus-galli (L.) Beauv.	2	2	2	1
Picris hieracioides L.	2	-	-	-
Portulaca oleracea L.	4	4	2	1
Rumex crispus L.	-	-	1	-
Senecio vernalis L.	1	-	1	-
Setaria viridis	1	2	-	1
*Silybum marianum**	-	1	-	-
Sinapis arvensis	-	1	-	-
Solanum nigrum	4	2	1	1
Sonchus arvensis	1	-	1	-
Stachys annua	1	-	-	-
Taraxacum officinale	1	1	1	-
Xanthium strumarium	-	1	-	-
Total	30	27	21	8

* Medical plants that are grown at the Experimental field of Institute of Medicinal Plant Research „Dr Josif Pančić"

DISCUSSION

There is a common perception that composting kills weed seeds. Namely, the temperatures which are reached during the composting phase are usually high enough to kill most weed seeds (De Luca & De Luca, 1997). Nishida et al. (1998) found that a base temperature above 46 °C seems to be required to achieve a significant reduction in seed viability, while Eghball and Lesoing (2000) considered that such temperatures are >60°C. For example, Dahlquist et al. (2007) found that the temperatures of 50°C and above were lethal for seeds of all six weed species (*Amaranthus albus* L., *Echinochloa crus-galli* (L.) Beauv., *Portulaca oleracea* L., *Sisymbrium irio* L., *Solanum nigrum* L., *Sonchus oleraceus* L.) they examined under controlled laboratory conditions. Contrary to that, in our experiment some seeds of some of the mentioned weed species (*E. crus-galli*, *P. oleracea*, *S. nigrum*) survived in all four treatments. Our results regarding the seed viability of four weed species (*Achillea millefolium*, *Bifora radians*, *Convolvulus arvensis*, *Stachys annua*) in all treatments, containing activators, are also contrary to the findings of Wiese et al. (1998), who found that *Convolvulus arvensis* L. was the most difficult species to eliminate during composting, in comparison to *E. crus-galli* and themixture of *Amaranthus* sp., *K. scoparia* and *Sorghum halepense*.

Aside from other factors which affect the seed survival during composting, species characteristics also play an important role in their survival. For example, Weise et al. (1998) found that seeds of *E. crus-galli* and the mixture of *Amaranthus* sp. and *Kochia scoparia* (L.) Schrad. were killed after three days at 49°C in composting manure, seeds of *Sorghum halepense* L.(Pers.) was killed with three or more days of exposure to 72 °C, while seeds of *Convolvulus arvensis* L. were killed only after seven days at 83 °C. Although, Tompkins et al. (1998) has observed a complete loss of seed viability in several weed species after windrow composting for 4 weeks, in our experiment some seeds of 28 species survived the composting during much longer periods. This concurrs with the findings of Ozores-Hampton et al. (1999), who confirmed that the duration of composting appears to be less important than the temperature.

In conclusion, we have found that different fertilizers, added as activators to compost prepared from biological waste, affected the germination of weed seeds. But, it hasn't been easy to estimate the effects of these activators on seed survival in the compost, because some other factors have also affected the seed survival during the composting process.

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THE POTENTIAL OF LOCAL POPULATIONS OF (*Encarsia formosa* Gahan) IN BIOLOGICAL CONTROL OF GREENHOUSE WHITEFLY (*Trialeurodes vaporariorum* Westwood) IN SERBIA

Tanja Drobnjaković*[1], Mirjana Prijović [1], Pantelija Perić [1] Slobodan Milenković [2] and Svetomir Stamenković [3]

[1] Institute of Pesticides and Environmental Protection, Banatska 31B, 11080 Belgrade-Zemun, Serbia
[2] Megatrend University, Faculty of Biofarming, MaršalaTita 39, 24300 BačkaTopola, Serbia
[3]PrištinaUniversity, Faculty of Agriculture, Lešak, Srbija
**tanjadrobnjakovic@gmail.com*

ABSTRACT

The present study investigated the biological parameters (development time, longevity, parasitism, adult emergence and instantaneous rate of increase) of females from two local populations of *Encarsia formosa* Gahan and a commercialized race of the parasitoid (Dutch strain).

The representative local populations of *E. formosa* were set up from pupae collected from vegetables and ornamentals grown in tunnel greenhouses in localities without a tradition in using commercial strains of the parasitoid for biological control of the greenhouse whitefly. The two local populations and the Dutch strain were reared on tobacco plants infested with whiteflies. The laboratory conditions included: t°C 26±2, RH 60±10% and L:D=16:8.

The data showed that parasitoid females of the local Bujanovac population had the best results of longevity (10.14±0.21 days), parasitism of the host (175.09±3.81 pupae/female), adult emergence (148.76±4.74 adults/female) and instantaneous rate of increase (0.332±0.002 female/day). Compared to the local Negotin population and Dutch strain females, those from Bujanovac had significantly higher statistical values of parasitism and adult emergence, which were the most important indicators of biological efficacy. The results make a starting point for further evaluation of local populations of the parasitoid as a potential biological agent.

Keywords: *Encarsia formosa*, development, reproduction, population growth

INTRODUCTION

The greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) has been widespread in Serbia since the 1970s and frequently found in greenhouses as one of the most serious pests of vegetables and ornamentals (Perić, 1999). As the widespread use of chemical insecticides has caused whitefly resistance to compounds of earlier generations, as well as some newer insecticides (Whalon et al., 2015), long-term and sustainable strategies for control of this pest species should be based on an integration of chemical, biological, cultural and other measures (Gentz et al., 2010).

The parasitic wasp *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae; Coccophaginae) is a uniparental, thelytokous endoparasitoid which has been used for many years for biological control of *T. vaporariorum* as one of the most successful biological agents in greenhouse and ornamental crops around the world (Lenteren and Martin, 1999). In a group that also includes *Encarsia tricolor* Foerster, *Encarsia partenopea* Masi and *Encarsia lutea* Masi, *E. formosa* has been identified as the most widespread of the four parasitoids of *T. vaporariorum*

and *Aleyrodis lonicerae* Walker on vegetables, ornamentals and many species of wild flora (Perić, 1999).

Many studies focusing on different aspects of the biology of a highly commercialized Dutch strain of *E. formosa* have been published so far (Arakawa 1982; Bethke et al., 1991). On the other hand, there have been few studies investigating the parasitoid's local populations reared on whitefly hosts (Hu et al., 2002). Our earlier research had focused on the potentials of local Serbian populations of the parasitoid *E. formosa* Gahan in control of the greenhouse whitefly and the most successful parasitoid population was one from the locality Bujanovac (Drobnjaković et al., 2013), while a population from Negotin locality had the lowest potential. In the present study, life history traits of these two local populations of *E. formosa* originating from different regions of Serbia were examined and compared to the Dutch strain.

As implementation of the principles of integrated control of whiteflies is in its initial stage in Serbia at present, data on parasitoid potentials of those local populations were intended to create a starting point for further research that would focus on improvement of local whitefly management programmes.

MATERIALS AND METHODS

The local populations of *E. formosa* were set up from pupae collected in tunnel greenhouses of vegetables and ornamentals in locations without a tradition in using commercial strains of the parasitoid for biological control of greenhouse whiteflies, namely; Bujanovac (B) on *Solanum nigrum* L. and Negotin (N) on *Hibiscus* sp. The emerged female wasps of each population were identified as *E. formosa* using the key given by Polaszek et al. (1992). The Dutch strain of *E. formosa* (D) was purchased from Zeleni hit d.o.o., the Serbian agent of Koppert Biological Systems Inc., The Netherlands, and successfully cultured as a reference strain.

The Dutch strain of *E. formosa* and two local populations of the parasitoid wasp were reared on *T. vaporariorum* hosts at 27±1°C and 60±10% R.H. with a 16L:8D h photoperiod. Whiteflies have been reared massively on tobacco plants, cv. Samsun, in ventilated muslin cages according to EPPO (2004) methodology.

All bioassays were carried out under 27±1°C, 60±10% R.H. and 16L:8D h photoperiod with four replications. To determine the time of development, five females of *E. formosa* per perforated plastic bag were released by fixing each bag around the petiole of a tobacco leave inside it that was infested with fourth-instar whitefly nymphs. After 24 h, the females were removed together with bags. When the parasitoids were on the verge of emerging from pupae, 12 h counting intervals began of monitoring their numbers (Enkegaard, 1993). The development time was calculated as the total number of days from the parasitoid's egg laying to adult emergence from pupae.

The female longevity test (in the presence of host, i.e. with host larvae offered for parasitism) was carried out by inserting 20 newly-emerged females of *E. formosa* (aged up to 24 h) in Petri dishes that contained leaves with host larvae laid upon 1% agar medium (Qiu et al., 2004). Two or three days later, females were transferred to new Petri dishes with hosts, checked daily and the transferring continued until the death of the last insect. Adult longevity was calculated as the total number of days that each female lived, assuming that females died at the midpoint of 24 h interval. The Kaplan-Meier analysis was used to estimate the longevity data (SPSS Statistics, Version 17) and survival curves (StatSoft, version 7).

To evaluate host parasitism, ten females of *E. formosa*, aged up to 24 h were inserted in Petri dishes with infested tobacco leaves laid upon agar medium and left to lay eggs over the next 48 h before they were transferred to new leaves with host larvae and kept until the death of the last insect. The number of parasitized hosts was determined when nymphs changed colour from light to dark. After counting, the parasitized host larvae (pupae) were transferred to new and clean Petri dishes to monitor adult emergence from pupa (fertility) (Stouthamer and Mak, 2002). Parasitism was calculated as the number of parasitized pupae per female alive at the midpoint of 48 h (parasitization rate) and summed over the female lifetime (total parasitism). Adult emergence was calculated as the total number of individuals reaching the adult stage from parasitized pupae. Parasitism and adult emergence data were transformed by $\sqrt{(x+0.5)}$. Parasitism and fertility data were used to calculate the instantaneous rates of increase(r_i) using the equation given by Walthall and Stark (1997) at the end of the 16th day after the start of oviposition.

The development times, longevities, parasitism and calculated r_i values of different populations were analyzed by one-way ANOVA with the means separated by Fisher's LSD test ($p<0.05$). The log-rank test was applied to compare survival rates (StatSoft, version 7).

RESULTS

Adults from all studied *E. formosa* populations began their massive emergence on the 14th day after the start of egg laying, and their development completed by the 18th day. The longest juvenile development time was found

in *E. formosa* population D (14.63±0.30), followed by population B (14.35±0.10 days). Population N had the shorest development time (14.28±0.25 days) but there were no statistically significant differences between the commercialised and local populations regarding the duration of juvenile development.

Females of the local *E. formosa* population B (10.14±0.21days) lived statistically significantly longer than those of population N (07.25±0.17 days) ($F_{2,9}$=13.979; p<0.01), but there were no statistically significant differences in adult longevity between local populations B and the commercialised population D (08.99±0.12 days).

The survival curves of the examined *E. formosa* populations are shown in Figure 1. The highest female survival was found in population B, followed by females of population D, but no significant difference was detected between these two (B vs. D: ww =-11.92;p<0.1). The females of population N had the lowest survivorship that differed significantly from females of the other two populations (B vs. N: ww = -23.5; p <0.001; D vs. N: ww = 15.366; p <0.001).

Table 1 shows parasitization rates of the examined *E. formosa* populations. In our experiment, females of the examined populations parasitized up to 54. 28% of all parasitized pupae within the initial six days of oviposition.

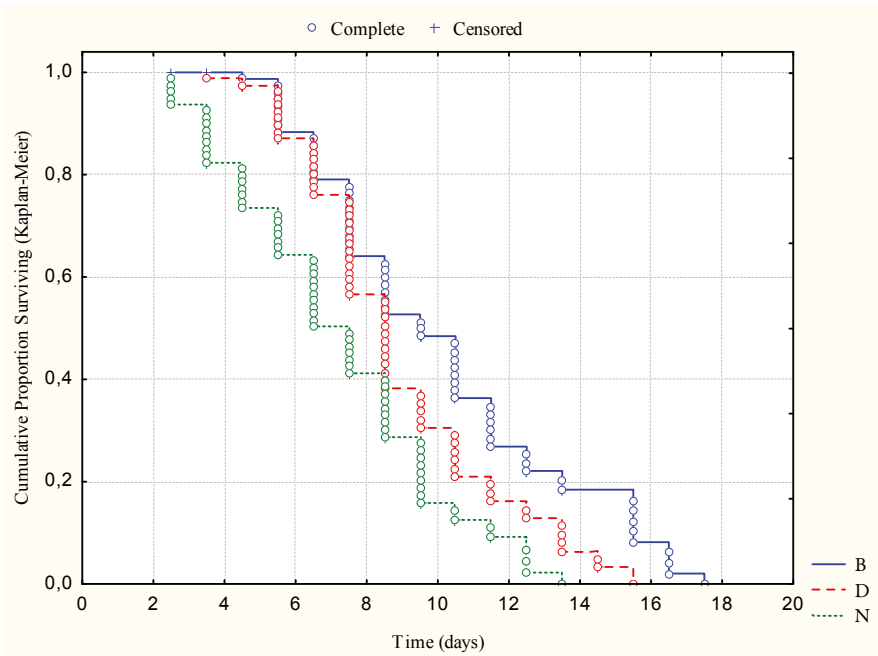

Figure 1. Survival curves of *E. formosa* females from populations Bujanovac (B), Negotin (N) and the Dutch strain (D) in the presence (B) of the host, third- or fourth-instars of *T. vaporariorum* nymphs

Table 1. Parasitization rates (means ± SE, pupae/female/48h) of *E. formosa* females from populations Bujanovac (B), Negotin (N) and the Dutch strain (D)

Population *E. formosa*	Oviposition (days)								
	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18
B	22.3b* (±0.44)	24.97ab (±0.43)	26.28a (±0.62)	22.42a (±0.64)	22.06a (±0.42)	16.2a (±0.88)	16.70a (±1.05)	13.92b (±0.65)	10.25 (±3.52)
D	18.44a (±0.79)	22.63a (±0.79)	25.77a (±0.85)	22.00a (±1.61)	20.74a (±0.63)	12.43a (±4.19)	7.27a (±4.21)	3.5a (±3.5)	-
N	18.08b (±1.03)	21.76b (±1.19)	16.75b (±1.60)	15.23b (±0.87)	13.23b (±1.25)	9.47a (±3.22)	9.75a (±3.57)	-	-

* The mean values with different letters in the same column are significantly different (ANOVA followed by Fisher LSD's test p<0.05).

The examined *E. formosa* populations showed statistically significant differences regarding their total parasitism ($F_{2,9}$= 29.079; p<0.001) and adult emergence ($F_{2,9}$=33.348; p<0.001). The highest values of those two life history traits, found in the females of population B (175.09±3.81 pupae/female and 148.76±4.74 adults/female), were significantly different from those of females in population D (132.77±8.20 pupae/female and 119.45±6.92 adults/female) and population N (104.26±6.13 pupae/female and 86.89±7.83adults/female).

The instantaneous rates of increase of *E. formosa* populations were calculated starting from a 16-day oviposition interval (the choice was based on an observation that massive emergence of adults from eggs laid on the first day occurred after 14 days) until the end of oviposition. At the end of oviposition, the highest r_i values were found in populations B (0.332 day^{-1}) and D (0.322 day^{-1}), significantly higher ($F_{2,9}$= 15.39; p<0.01) than in population N. The values of r_i found in population N (0.296 day^{-1}) were 1.12- and 1.09-fold lower than the r_i values in populations B and D, respectively.

DISCUSSION

Similar data regarding the development time of *E. formosa* on *T. vaporariorum* hosts have been reported from other studies. Studying a local Serbian population reared on beans, Perić (1999) had found that juveniles required 14.8 days to complete their development at 27°C. Arakawa (1982) had earlier found the Dutch strain of *E. formosa* to have developed for 15 days on tobacco plants at 25°C, while the strain had developed 14 days on pepper, aubergine and cucumber at 22.5-25°C (Woetsand Lenteren, 1976).

Perić (1999) had reported that females of a local population of *E. formosa* lived 13.1 days on the average at 27 °C on beans. Such considerable differences may be attributed to the different methodologies applied (different ways of offering hosts to parasoid females, and different host plants). Madueke (1979) had reported a similar longevity of *E. formosa* Dutch strain of 11.4 days (at 27°C on beans).

Studying a local population in Serbia at 27°C, Perić (1999) had recorded higher values of total parasitism of around 200 pupae/female. Total parasitism of population D females in our experiment was lower than the total parasitism reported by Madueke (1979), 160.02 pupae/female at 22.5°C on bean plants. Since oviposition duration may affect total parasitism, when total parasitism was calculated for a period of 14 days (to cancel the effect of different oviposition durations) this parameter in females of population B (144.10±4.44 pupae/female) still had significantly higher statistical values than females from local and commercial population D ($F_{2,9}$=13.573; p<0.01).

Significant differences in adult emergence resulted mostly from the total parasitism of populations because the proportion of total emerged adults was similar, ranging from 84% to 90%.

The instantaneous rate of increase is an alternative measurement of population growth that integrates both survivorship and reproduction, yet it is not as time- and labour-consuming as life table bioassays (Walthall and Stark, 1997).

The data acquired in the present study show that females of the local *E. formosa* population B demonstrated the most promising results for integrated control of whitefly in Serbia, compared to the Dutch strain. The results provide a starting point for further evaluation of the *E. formosa* local population B as a biological agent for control of *T. vaporariorum* in Serbia. Further studies should focus on the genetic origin of local populations, their efficacy in practical uses, and evaluation of insecticidal effects on their biological parameters.

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ACARICIDAL AND BEHAVIORAL EFFECTS OF AZADIRACHTIN ON TWO-SPOTTED SPIDER MITES (Acari: Tetranychidae)

Irena Međo [1]*, Dejan Marčić [1] and Slobodan Milenković [2]

[1]Institute of Pesticides and Environmental Protection, Banatska 31B, 11080 Belgrade, Serbia
[2]Megatrend University, Faculty of Biofarming, Maršala Tita 39, 24300 Bačka Topola, Serbia
**corresponding author e-mail: irenao79@gmail.com*

ABSTRACT

The triterpenoid azadirachtin, a major active ingredient of products derived from seeds of the Indian neem tree (*Azadirachta indica*), is one of the most widely sold botanical insecticides. Azadirachtin has been shown to possess acaricidal properties as well, but its effects on spider mites varied with product and formulation type. The objective of this study was to evaluate toxic and behavioral effects of the botanical pesticide NeemAzal-T/S, an emulsion concentrate containing 10 g/l of azadirachtin-A, on the two-spotted spider mite, *Tetranychus urticae* Koch, in order to obtain data that could be used in further research aimed to improve the management of this important pest. The botanical pesticide was applied to bean leaf discs (3 cm diameter) or primary leaves positioned on moistened cotton wool in Petri dishes by a Potter spray tower. Its effects on eggs, larvae, protonymphs, female deutonymphs, female teleiochrysales, and preovipositional adult females were tested in several successive acute toxicity bioassays in 4-8 replicates. Acaricidal activity, based on the number of treated individuals reaching the adult stage, was assessed in bioassays with eggs and immatures. In an adult female bioassay, biological response (mortality-runoff rate) based on the numbers of live females and runoff females was assessed 24 h and 72 h after treatment. Concentration-response data were subjected to Probit analysis (POLO Plus, LeOra Software). The following EC_{50} and EC_{90} values were calculated (in mg/l a.i.): 7.62 and 17.45 (eggs), 5.15 and 9.51 (larvae), 5.86 and 11.56 (protonymphs), 11.55 and 33.59 (female deutonymphs), 35.40 and 92.43 (female teleiochrysalises), 14.67 and 74.38 (female adults, after 24 h), 6.42 and 29.12 (female adults, after 72 h). Concentration-dependent run-off effect was observed in the bioassay with female adults (10-76% and 17-81% after 24h and 72h, respectively).

Keywords: *Tetranychus urticae*, azadirachtin, toxicity, behavior

INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is a highly polyphagous and cosmopolitan pest species possessing a remarkable intrinsic potential for rapid evolution of resistance to synthetic chemical acaricides. Acaricide resistance in *T. urticae* (and several other spider mite species) has become a global phenomenon and created a permanent need for development of novel compounds with acaricidal action (Dekeyser, 2005; Van Leeuwen et al., 2010; Marčić, 2012). On the other hand, increasingly rigorous regulatory criteria for introduction of plant protection products has reactualized the importance of biopesticides, usually viewed as agents with low risk to human health and the environment. Among

biopesticides of botanical origin there have been products representing potentially promising alternatives to synthetic compounds in the management of spider mite populations (Copping and Menn, 2000; Chandler et al., 2011; Marčić, 2012; Regnault-Roger et al. 2012; Attia et al., 2013).

Azadirachtin, a triterpenoid found in seeds of the neem tree, *Azadirachta indica* (Meliaceae), is one of most successful botanical pesticides, effective against a broad range of insect pest species. Azadirachtin- and neem-based products have been evaluated against spider mites as well. Laboratory experiments have revealed acaricidal action against *T. urticae* that varied with production technology and formulation type (Dimetry et al., 1993; Mansour et al., 1993, 1997; Sundaram and Sloane, 1995; Kashenge and Makundi, 2001; Knapp and Kashenge, 2003; Martinez-Villar et al., 2005; Duso et al., 2008). The biopesticide NeemAzal-T/S, a second generation product, is a formulation of highly concentrated and standardized extract of *A. indica* kernels (free of neem oil), containing azadirachtin-A as the leading active ingredient (Kleeberg, 2004). Only a few laboratory studies have so far focused on the acaricidal action of NeemAzal-T/S against two-spotted spider mites. Mironova and Khorkhordin (1997), Abdel-Aziz and Kelany (2001) and Dimetry et al. (2008) tested its acute toxicity to eggs and females. Dabrowsky and Seredyńska (2007) evaluated in two separate bioassays the behavior of *T. urticae* females exposed to NeemAzal-T/S and its toxicity to females. The objective of our present study was to obtain more baseline data regarding the biological effects of this product on different life stages of *T. urticae*, which could be used for further research aimed at improving the management of two-spotted spider mite populations.

MATERIALS AND METHODS

Spider mite population

A population of *T. urticae* set up from mites collected from a ruderal weed habitat on the outskirts of Belgrade has been reared on bean plants in a climate controlled room (25-30°C, 16/8 h of light/dark photoperiod) since March 2004.

Biopesticide

The commercial product NeemAzal-T/S (manufactured by Trifolio-M GmbH, Germany) is a formulation of NeemAzal, a highly concentrated extract of neem kernels free of neem oil, containing 34% of azadirachtin-A and around 20% of other isomers. NeemAzal-T/S is formulated as an emulsifiable concentrate containing a vegetable oil and surfactants produced from renewable resources. The content of azadirachtin-A in the product is standardized to 1% (10 g a.i./l). The concentration recommended for field application of NeemAzal-T/S against spider mites is 0.5% (50 mg a.i./l).

Bioassays

All bioassays were carried out on bean leaf discs (30 mm) or primary leaves positioned upon moistened cotton wads in Petri dishes with the abaxial surface upward. The Petri dishes were kept in a climate controlled room at 27±1.5°C, under 50-70% RH and 16/8 h of light/dark photoperiod. The biopesticide NeemAzal-T/S suspended in distilled water was applied to the discs/leaves by a Potter spray tower (2 ml of spray liquid, 100 kPa air pressure, aqueous deposit 2.7 ± 0.2 mg/cm^2). Its toxicity to eggs, larvae, protonymphs, female deutonymphs, female teleiochrysalises, and adult females of *T. urticae* was tested in several successive bioassays by spraying at least five serially diluted concentrations covering the range of 10-90% response. Control treatments were sprayed with distilled water alone. Egg bioassay was carried out in eight replications on primary bean leaves carrying 30-40 eggs (24 h old) per leaf. Bioassays with immatures were carried out in four replications on leaf discs with 20 mites per disc. Mortality was assessed based on the number of treated individuals (eggs, larvae, protonymphs, female deutonymphs) reaching the adult stage or the number of live adult females 48 h after treatment of female teleiochrysalises. The bioassay with adult females was carried out in four replications with 20-25 newly emerged (pre-ovipositional) females per disc and 1-2 discs per replication. Live females were counted 24 h and 72 h after spraying, as well as the females found trapped in surrounding cotton wads which had escaped the discs (runoff females). Runoff effect was calculated as the percentage of runoff females out of a total number of treated females. Biological response (runoff-mortality rate) was assessed based on the pooled number of runoff females and live females, related to the number of treated females.

Concentration-response data were subjected to Probit analysis using the POLO Plus software (LeOra Sotfware, Berkeley, CA). A pairwise comparison of lethal (LC) and effective (EC) concentrations at the 50% and 90% levels was performed using the lethal dose ratio test. When the 95% confidence limits for the ratio included 1 the LC/ECs were not significantly different (Robertson et al. 2007).

RESULTS AND DISCUSSION

The azadirachtin-based biopesticide NeemAzal-T/S exhibited various levels of toxicity to different life stages of *T. urticae* (Table 1). Larvae and protonymphs had the lowest LC_{50} and LC_{90} values among the pre-adult stages. Female deutonymphs were less susceptible than larvae and protonymphs, and female teleiochrysalises were the least susceptible pre-adult stage to the toxic action of NeemAzal-T/S. The acaricidal effect of direct treatment of eggs was actually the consequence of a residual toxicity to the larvae that hatched from those treated eggs (the percents of unhatched eggs in treatments were negligible and close to control value). This residual effect was lower than the toxic effect on directly treated larvae. Similar results regarding the ovicidal action of NeemAzal-T/S against *T. urticae* had been reported by other authors. Mironova and Khorkhordin (1997) found that the biopesticide, applied at 10 mg a.i./l and 30 mg a.i./l rates, caused virtually no mortality, while a concentration of 50 mg a.i./l produced 27% mortality of treated eggs. Abdel-Aziz and Kelany (2001) reported 13% mortality when eggs were treated with 50 mg a.i./l. Testing another azadirachtin-based product, Castagnoli et al. (2005) and Duso et al. (2008) found no significant egg mortality after application of the recommended concentration of 45 mg a.i./l. On the other hand, Dimetry et al. (2008) achieved a considerable ovicidal effect on *T. urticae* eggs when NeemAzal-T/S was applied at higher concentrations as the LC_{50} and LC_{90} were 47 mg a.i./l and 1230 mg a.i./l, respectively. Although the application techniques (and therefore the amounts of a.i. per cm^2) were different in all these studies, their results indicated a poor ovicidal action of azadirachtin applied at the recommended concentrations.

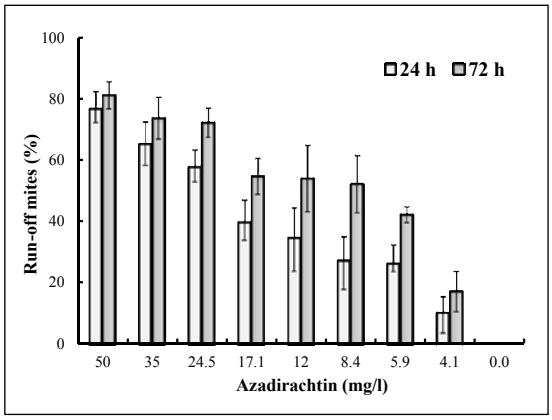

Fig. 1. The runoff response of *T. urticae* females after treatment with azadirachtin (mg/l)

Treated directly with NeemAzal-T/S, *T. urticae* adult females responded in two ways: a part of the females remained on leaf discs, while the other escaped the discs (runoff females). The runoff effect was concentration-dependent and ranged 10-76% and 17-81% after 24h and 72h, respectively (Figure 1). Most runoff females left the discs within the first 24 h, especially after the

Table 1. Acaricidal activity of azadirachtin (mg/l) to *Tetranychus urticae* after treatment of eggs (E), larvae (L), protonymphs (PN), female deutonymphs (DNf) and female teleiochrysalises (THf)

Life stage	n	LC_{50} (mg/l) (95% CLs)	LC_{90} (mg/l) (95% CLs)	b (± SE)	χ^2	df
E *	2067	7.62 b (7.18-8.05)	17.45 b (16.23-18.97)	3.56 (± 0.17)	3.94	4
L *	640	5.15 a (3.35-6.14)	9.51 a (7.80-17.10)	4.81 (± 0.75)	10.85	5
PN *	560	5.86 a (5.06-6.64)	11.56 a (9.64-15.96)	4.35 (± 0.51)	4.14	4
DNf *	390	11.55 c (9.86-13.32)	33.59 c (26.84-47.35)	2.76 (± 0.34)	1.48	4
THf **	460	35.40 d (27.55-55.82)	92.43 d (57.75-375.03)	3.08 (± 0.35)	6.37	3

* mortality was assessed based on the number of treated eggs/immatures reaching adult stage
** mortality was assessed based on the number of live females 48 h after treatment
LC values followed by different letters differ significantly (ratio test, P=0.05, Robertson et al., 2007)
n = number of treated eggs/immatures; CLs = confidence limits; b = slope of regression line; df = degrees of freedom

higher concentrations were applied. Runoff effects exceeding 50% were observed 24 h after the three highest concentrations were applied, while they were below 50 % only for the two lowest concentrations after 72 h. Azadirachtin is a well-known behavior-modifying agent for a number of insect species (Mordue /Luntz/ et al., 2010). Its runoff (repelent) effect on spider mite females has been observed as well (Mansour et al., 1993, 1997; Sundaram and Sloane, 1995; Kashenge and Makundi, 2001; Knapp and Kashenge, 2003; Brito et al., 2006). The extent of this effect has varied depending on azadirachtin product and concentration, and has often been recorded concomitantly with various levels of mortality among mites that remained on treated leaves. However, in other studies reporting mortality of *T. urticae* adult females treated with NeemAzal-T/S (Mironova and Khorkhordin, 1997; Abdel-Aziz and Kelany, 2001; Dimetry et al., 2008) such repelled or runoff mites have not been mentioned at all, even though the same leaf disc technique was used that gives mites a chance to leave treated surface. Unless ignored, such lack of runoff effect may be due to the fact that different populations/strains were involved.

In this study, we evaluated the response of *T. urticae* females to azadirachtin as a pooled runoff-mortality rate, considering runoff as the major effect and mortality as minor effect (with female mortality at maximum 20%). Some previous studies dealing with behavioral effects of pyrethroids and some other acaricides on spider mites (Fisher and Wrensch, 1986; Knight et al., 1990) had shown that mites leaving the treated plant surface would afterwards either be killed when reaching another contaminated plant surface or die of starvation away from a host plant. The runoff response should be therefore considered as a part of total mortality (or biological effectiveness) together with the mites killed by treatment directly.

Table 2 presents our estimates of the effective concentrations causing runoff-mortality response of *T. urticae* adult females to azadirachtin. Looking at the LC and EC values obtained in this study (Tables 1 and 2), and assuming a uniform distribution of azadirachtin, the recommended field rate of NeemAzal-T/S (50 mg a.i./l = 135 ng a.i./cm^2) should be expected to eliminate the tested population almost completely. Only a small part of the females treated at the last quiescent stage (teleiochrysalises) were able to survive. However, spray coverage is heterogenous in the field, often leaving small uncovered areas (refugia) that could be colonized by spider mites surviving treatment (Sáenz-de-Cabezōn Irigaray & Zalom, 2009; Martini et al., 2012). From that point of view, the concept of total mortality should yield to the concept of population recovery potential, which is directly dependent on mites' ability to find and reach refugia, i.e. on females surviving treatment as teleiochrysalises or young adults. Marčić and Međo (2015) have recently reported that *T. urticae* females surviving treatment with field relevant rates of NeemAzal-T/S during their preovipositional period retained a significant reproductive potential (females were sprayed as newly emerged adults and after 24 h of residual exposure to the biopesticide transferred to untreated leaf discs). Having considered these and all other published data on the acaricidal effects of NeemAzal-T/S against *T. urticae*, we inferred that a further laboratory and field research is needed to improve the use of this product against two-spotted spider mites.

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Table 2. Biological response (mortality+runoff)* of *Tetranychus urticae* adult females (n = 1360) after treatment with azadirachtin (mg/l)

Time after treatment	EC_{50} (mg/l) (95% CLs)	EC_{90} (mg/l) (95% CLs)	b (± SE)	χ^2	df
24 h	14.67 b (11.70-17.79)	74.38 b (53.30-126.42)	1.82 (± 0.12)	14.09	6
72 h	6.42 a (4.09-8.55)	29.12 a (22.27-43.83)	1.95 (± 0.14)	18.31	6

* assessed based on a pooled number of live and runoff females
EC values followed by different letters differ significantly (ratio test, P=0.05, Robertson et al., 2007)
n = number of treated pre-ovipositional adult females; CLs = confidence limits; b = slope of regression line; df = degrees of freedom

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EVALUATION OF AQUEOUS EXTRACTS FROM NATIVE PLANT SPECIES FOR THEIR NEMATICIDAL EFFECTS ON *Meloidogyne* spp.

Lamia Tafifet, Zoulikha Krimi and Dhaouya Nebih Hadj-Sadok

Laboratoire de phytobactériologie, Département des Biotechnologies, Faculté des Sciences de la Nature et de la Vie, Université de Blida 1. BP 270, route de Soumaa, Blida Algérie.
E-mail: krimizlk@yahoo.fr

ABSTRACT

Aqueous extracts of four dominant botanical species; *Calendula arvensis, Euphorbia helioscopia, Plantago lanceolata* and *Urtica dioica* collected from a fallow field compared to a synthetic nematicide (Vydate) were tested for their toxicity against the root knot nematode *Meloidogyne* spp. Results of *in vitro* of the tests showed a nematicidal activity resulting in an increased mortality of the second stage larvae. The mortality was dependent on the increased concentration and the exposure period to treatments. Undiluted extracts from the four plant species and the chemical nematicide exhibited an amount of mortality of juveniles larvae more than 80%. Larval mortality decreased with an increase in the dilution for all the aqueous plant extracts and similarly with an increase in exposure time, juvenile mortality was also increased.

Indeed, *Urtica dioica* was the most toxic plant compared to the other species and to Vydate with a mortality of juveniles reaching 100%, revealing a high shock effect after 6 hours.

The calculation of lethal concentration 50 for each plant extract showed a significant toxicity of Urtica *dioica* and *Euphorbia helioscopia* aerial extracts parts with 20.80 mg/cm^3 and 35.19 mg/cm^3 respectively and a low nematicidal effect with 351.80 mg/cm^3 for *Calendula arvensis* roots.

Keywords: Aqueous plant extract, *Meloidogyne* spp., larval mortality, nematicidal effect.

INTRODUCTION

Plant-parasitic nematodes belonging to *Meloidogyne* spp. represent one of the most important group of pests and the most dangerous to crops. They are parasitic to more than 2,000 plant species and are found throughout the globe (Azhagumurugan and Rajan, 2014). Nematodes cause deformations on the root system and infested crops record yield losses of up to 80 % in heavily contaminated soils. In Algeria, several studies showed the importance of vegetable crops infestations by *Meloidogyne* spp. both in coastal areas and in Sahara zones where rates of infestations ranged from 49% to 100% (Sellami et al., 1999). Chemical control is still the most used method, but, is not able to solve the infestation of *Meloidogyne* spp. populations (Nebih et al., 2011). Populations of plant-parasitic nematodes in the field can be controlled through several approaches such as using natural enemies, enhancing cultural practices, cultivating resistant cultivars and applying pesticides. However, the excessive use of chemicals to control important damages caused by nematodes on cultivated plants resulted in ecotoxicological issues that were clearly recognized in these recent decades. Due to the adverse effects of chemical nematicides on the environment, many have been or are currently being withdrawn from the market (Onkendi et al., 2014). Environmentally safe alternative methods of control are being proposed and many botanicals have been identified as presenting a biopesticide activity on plant parasitic nematodes. These biopesticides are considered to be non-persistent under field conditions and without residues in the environment.

The objective of the present study was to evaluate the effectiveness of some native plant species on juveniles *Meloidogyne* spp.

MATERIAL AND METHODS

Preparation of plant extracts

Four spontaneous plant species; *Calendula arvensis* L., *Euphorbia helioscopia* L., *Plantago lanceolata* L. and *Urtica dioica* L. randomly collected from a fallow field in the Mitidja area were screened for their nematicidal effect. Collected samples of fresh leaves and roots of each plant species were separately washed thoroughly in tap water, air dried and powdered with a blender. The extraction process used in this experiment was the aqueous maceration. Cold water extract solutions were prepared by suspending the powder extracts (20g in 250ml of distilled water) for 72h. Suspensions were then filtered through a Whatman filter paper n°1 and stored in dark at 4°C. Aqueous extracts were used undiluted and diluted 75 and 50 times with distilled water.

Extraction of juvenile nematodes

Root knot nematodes were maintained on infested tomato plants in pots under greenhouse conditions. Second stage larvae were extracted according to the method described by Whitehead and Hemming (1965).

Effect of plant extracts on juvenile larvae mortality

Suspensions of second stage juveniles containing 20 larvae counted under an inverted microscope were deposited in cell culture dishes. A volume of 1ml of the pure plant extracts (pure: undiluted) and their dilutions (50: diluted 50 times and 25: diluted 75 times) for each plant part (shoots, roots) was added separately to the suspensions of larvae. Dishes containing distilled water and a chemical nematicide Vydate (15 µl/ml by dish) were used respectively as negative and positive controls. All treatments were replicated four times. Dishes were then incubated at 25°C and recorded under an inverted microscope for the percentage of mortality after 24, 48 and 72 hours under exposure to the treatment.

The shock effect was performed for the extract of the plant species which gives a high level of mortality. While recording the data on nematode mortality, the lethal effect (nematicidal) was distinguished from the nematostatic effect (suppression of nematode movement). The extract solution in cell dishes was removed and washed three times with distilled water and numbers of dead/live nematodes were recorded after 24h.

Table1. Effect of plant extracts and Vydate on mortality of *Meloidogyne* larvae.

Treatment	Exposure time	Extracts Dilutions					
		Arial part			Roots		
		Pure	50	25	Pure	50	25
E. helioscopia	24h	25*	25	5	30*	10*	5*
	48h	30*	10	5	35*	20*	10*
	72h	80*	50*	30*	90*	55*	30*
P. lanceolata	24h	45*	30*	15*	35*	10	5
	48h	65*	35*	20*	40*	10*	5
	72h	80*	60*	40*	55*	15*	5
C. arvensis	24h	30*	35*	20*	0	0	0
	48h	35*	40*	20*	5*	10*	5
	72h	70*	45*	20*	20*	10*	5
U. dioica	6h	55*	25*	5	35*	10	0
	24h	40*	50*	45*	40*	45*	40*
	48h	95*	85*	45*	80*	50*	35*
	72h	100*	90*	50*	80*	65*	40*

		Pure	50	25
Vydate	6h	5	5	0
	24h	80*	80*	65*
	48h	85*	85*	80*
	72h	95*	90*	85*

*P<0,05 (significant). Numbers represent the average value of percentage of larval mortality.
** Recommended rate: 15µl/ml by dish.

All the data collected were statistically treated by using Analysis of Variance and the Global Linear Model (GLM). The mean differences between the treatments, concentrations of the extracts and plant parts (shoots, roots) and the time of exposure in terms of percentages of mortality were determined by Analysis of Variance (Dagnelie, 2007). The mean difference of the extracts and the plant parts was analyzed by the Global Linear Model GLM at $P < 0.05$. The efficacy of a treatment is measured by the lethal concentration 50 (in 50%) which is deducted from the regression line (Chan and Hayes, 1989).

RESULTS

The four plant extracts and the synthetic chemical compound (Vydate) proved qualitatively and quantitatively efficiency against root-knot nematodes resulting in an increased mortality of second stage juveniles according to the increased period of exposure to treatments. Compared to the negative control, no mortality was recorded after 72h. Extracts of the aerial parts (shoots) gave very highly significant effects resulting in a mortality of nematodes exceeding 70% compared to the roots which gave less than 50% (table 1).

Nematicidal activity of aqueous extracts from the four plant species studied on larvae, varied significantly depending on the plant species, the compartment and the time of exposure to treatment (Fig. 1). Aqueous extracts of *Urtica dioica* were the most toxic 24h and 48h after treatment and produced a very high mortality (75%) compared to the other plant species and to Vydate. The Vydate proceeded quickly and induced a high mortality on larvae after 24h of exposure and this result is independent on the used concentration. The nematicidal activity of extracts from *P. lanceolata* and *E. helioscopia* increased with exposure time and revealed a mortality rate up to 55% after 72h, however, extracts from *C. arvensis* exhibited the lowest toxicity (30% of mortality). Extracts from the shoots were very significant (P=0.000), the mortality exceeded 70% after 72h of treatment compared to the roots that didn't exceed 50%. After 72h of exposure to the different treatments, the percentage of regeneration was less than 17%, nematodes were immobile, exhausted and could no longer move and roll up compared to the water control where they exhibited activity and mobility. These last results confirmed a nematicidal and not a nematostatic effect of the plant extracts. Results of lethal concentration 50 for *Urtica dioica* and *Euphorbia helioscopia* aerial extracts were respectively 20.80 mg/cm^3 and 35.19 mg/cm^3, however, *Calendula arvensis* roots were less efficient (351.80 mg/cm^3).

Cal: *Calendula arvensis,* **Eup**: *Euphorbia helioscopia,* **Urt***: Urtica dioica,* **Plan**: *Plantago lanceolata,* **V***:* Vydate, **A**: Arial part, **R**: roots, **P**: probability (P<0,05).

DISCUSSION

Plant extracts from species such as *U. dioica*, *P. lancelolata* and *E. helioscopia* are more likely to reduce plant-parasitic nematode (*Meloidogyne* spp.) populations and strongly suggest the presence of nematicidal substances such as phenolic compounds and terpenoids in the whole plant and the possibility of using the plant species for control (Stavrianakou et al., 2005).

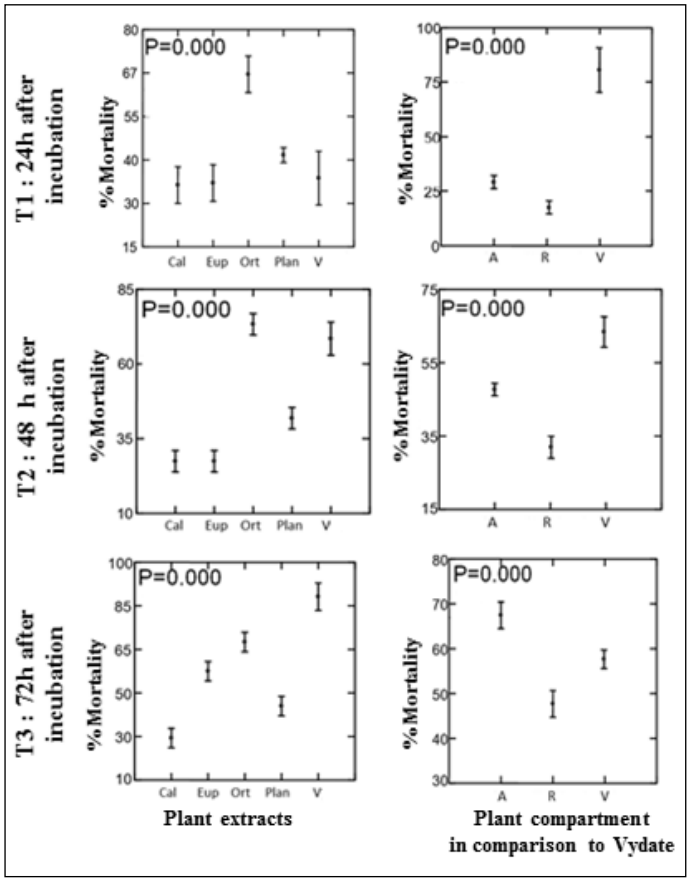

Figure 1. Toxicity of aqueous plant extracts and Vydate according to the time of exposition to the treatments.

The differences in toxicity of the different extracts could be attributed to the presence of active compounds in the plant material that may be influenced by several factors such as age of the plant, method of extraction and type of extracting solvent. Nematode mortality was strongly influenced by concentration of extract, plant species and duration of exposure.

Typically, shoot extracts were more efficacious compared to root extracts; this finding which agrees with the results reported by other studies, could be explained by the fact that the toxic compounds were better solubilised in the acqueous extracts from the shoots than the roots (Agbenin et al., 2005). Results of the present study indicate that the tested plant extracts reveal a nematicidal activity and suggest that they are useful to economically biocontrol populations of parasitic *Meloidogyne*. The application of these plant species as a botanical pesticide for future use against nematodes is highly promising especially in green farming and organic production but there is a need for further study especially in assessing the effectiveness of plant extracts in field environment.

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THE EFFECT OF *Thymus serpyllum* L. AQUEOUS EXTRACT ON A BROMUS SEEDLINGS

Jovana Šućur[1], Dejan Prvulović[1], Đorđe Malenčić[1], Goran Anačkov[2] and Milan Popović[1]

[1]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad
[2]Faculty of Science, University of Novi Sad, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia
E-mail:jovanasucur@yahoo.com

ABSTRACT

Due to an increase in the number of herbicide-resistant weeds and environmental concerns in the use of synthetic herbicides, there have been considerable efforts in designing alternative weed management strategies. The aim of this study was to examine the effect of the aqueous extract of *Thymus serpyllum* L. on bromus antioxidant properties so as to explore the properties of these species in the control of weeds. Changes in antioxidant systems in leaves and roots of bromus (*Bromus mollis* L.) treated with two different concentrations of *T. serpyllum* aqueous extracts (0.1 % and 0.2 %) were studied 24, 72 and 120h after the treatment. The generation of superoxide and hydroxyl radicals was evaluated together with the production of malonyldialdehyde, the main end product of lipid peroxidation (LP). Two enzymatic parameters were monitored as well, the activity of antioxidant enzymes pyrogallol and guaiacol peroxidases. Our results show that both concentrations of *T. serpyllum* aqueous extract induce lipid peroxidation in bromus roots. The highest level of MDA was observed in roots treated with the lower concentration of *T. serpyllum* aqueous extract, 24h after the treatment. In the bromus leaves, a significant increase in the LP intensity was recorded in plants treated with the lower concentration of *T. serpyllum* aqueous extract, 120 h after the treatment. Furthermore, significant increases of antioxidant enzymes were detected in bromus roots treated with 0.1 % *T. serpyllum* aqueous extract 24h after the treatment.

Keywords: *Thymus serpyllum L; Bromus mollis* L.; allelochemicals; biopesticides

INTRODUCTION

A large number of plants can release chemicals into the environment that suppress the germination and growth of neighboring plants (Inderjit et al., 2011; Kato-Noguchi and Ino, 2005). This phenomenon is known as allelopathy (from the Greek *allelon* = of each other, *pathós* = to suffer) (De Albuquerque et al., 2010). Since allelopathy is definided as direct influence of chemical (allelochemicals) released from one plant on the development of other plants, many investigations have been attempted to exploit allelopathy of plants for weed control purposes (Kato-Noguchi, 2004).

Thymus species (Lamiaceae) are considered as medicinal plants due to their pharmacological and biological properties (Hussain et al., 2013). In the Mediterranean region, which can be described as the centre of this genus, annual aromatic herb, *Thymus serpyllum* L. (wild thyme) inhabits cultivated and uncultivated lands including wastelands (Verma et al., 2011). Thymus oils and extracts are widely used in pharmaceutical, cosmetic and perfume industry also for flavouring and bio-preservation of several food products (Verma et al., 2011).

The aim of this study was to examine the effects of aqueous extract of *T. serpyllum* L. on bromus antioxidant properties and to explore the potential of this species in weed control.

MATERIAL AND METHODS

The wild, aromatic plant, *Thymus serpyllum* L., was collected in northern Serbia (Vojvodina province) in May of 2010. Voucher specimens of collected plant was confirmed and deposited at the Herbarium of The Department of Biology, Faculty of Natural Sciences, University of Novi Sad.

The aqueous extract of *T. serpyllum* was prepared with air-dried plant material (10 g) in boiling distilled water (100 ml). After 24 h, the extract was filtered through filter paper and kept at 4 °C until application.

The bromus (*Bromus mollis* L.) seeds were grown in a controlled climate chamber at 28 °C, 60% relative humidity, a photoperiod of 18 h, and a light intensity of 175 μmol m^{-2} s^{-1}, in plastic pots containing sterile sand. After 30 days, the plants were transplanted in plastic pots containing 700 ml of Hoagland's solution and 7 and 14 ml of *T. serpyllum* aqueous extract, while pots of control contained the same volume of Hoagland's solution. Plants were harvested for further biochemical analyzes 24, 72 and 120 h after the treatments.

Fresh leaves and roots of bromus plants (2 g each) were homogenized in 10 ml of phosphate buffer (0.1 M, pH 7.0). Homogenates were centrifuged for 20 min at 10.000 x *g* and filtered. The supernatants were used to test enzyme activity and to determine intensity of lipid peroxidation.

Lipid peroxidation (LP) was measured at 532 nm using the thiobarbituric acid (TBA) test. The total amount of TBARS (TBA-reactive substances) is given as nmol malondialdehyde (MDA) equivalents mg^{-1} proteins (Mandal et al., 2008).

Peroxidase (EC 1.11.1.7) activity was measured using guaiacol (guaiacol peroxidase; GPX) and pyrogallol (pyrogallol peroxidase; PPX) as substrates according to Morkunas and Gmerek (2007). Peroxidase activity (GPX and PPX) was expressed as U per 1 g of protein (U g^{-1} protein).

Values of the biochemical parameters were expressed as means ± standard error of determinations made in triplicates and tested by ANOVA followed by comparison of the means by Duncan's multiple range test ($P<0.05$). Data were analyzed using STATISTICA for Windows version 11.0.

RESULTS

Our results showed the significant increase of peroxidases activity in the roots of bromus plants treated with 0.1% *T. serpyllum* aqueous extract 24 h after the treatment (Table 1). After 72 and 120h, decrease of peroxidases activities were recorded in bromus roots treated with both concentrations. Also, in the leaves of the bromus plants, significant decrease in the activities of peroxidases were detected (Table 2).

Table 1. Effect of two concentrations of *Thymus serpyllum* aqueous extracts on activities of GPX and PPX (U g^{-1} protein) and on MDA content (nmol mg^{-1} protein) in roots of bromus seedlings

Time	24h	72h	120h
PPX			
Control	1.23 ± 0.01^{a}	3.04 ± 0.14^{d}	4.42 ± 0.02^{e}
0.1 %	5.05 ± 0.28^{f}	2.71 ± 0.21^{cd}	1.18 ± 0.06^{a}
0.2 %	0.70 ± 0.04^{b}	2.47 ± 0.10^{c}	0.60 ± 0.07^{b}
GPX			
Control	3.22 ± 0.05^{a}	25.34 ± 0.04^{g}	4.12 ± 0.58^{h}
0.1 %	13.30 ± 1.07^{e}	14.89 ± 0.33^{f}	9.46 ± 0.14^{d}
0.2 %	0.61 ± 0.03^{b}	7.73 ± 0.27^{c}	3.76 ± 0.17^{a}
LP			
Control	1.33 ± 0.10^{a}	1.32 ± 0.10^{a}	2.55 ± 0.19^{c}
0.1 %	4.56 ± 0.11^{e}	2.20 ± 0.05^{b}	2.70 ± 0.00^{c}
0.2 %	$2.45 \pm 0.05^{b,c}$	2.65 ± 0.06^{c}	3.06 ± 0.01^{d}

The data are mean values ± standard error. $^{a-h}$ values without the same superscripts within each column differ significantly ($P < 0.05$) PPX, pyrogallol peroxidase; GPX, guaiacol peroxidase; LP, lipid peroxidation

Table 2. Effect of two concentrations of *Thymus serpyllum* aqueous extracts on activities of GPX and PPX (U g^{-1} protein) and on MDA content (nmol mg^{-1} protein) in leaves of bromus seedlings

Time	24h	72h	120h
PPX			
Control	0.23 ± 0.00^{a}	0.33 ± 0.00^{c}	0.22 ± 0.00^{ab}
0.1 %	0.20 ± 0.00^{b}	0.12 ± 0.00^{e}	0.10 ± 0.00^{e}
0.2 %	0.16 ± 0.01^{d}	0.31 ± 0.01^{c}	0.10 ± 0.01^{e}
GPX			
Control	0.65 ± 0.03^{a}	1.67 ± 0.04^{f}	1.12 ± 0.02^{d}
0.1 %	0.22 ± 0.01^{e}	0.26 ± 0.01^{e}	1.05 ± 0.01^{c}
0.2 %	0.23 ± 0.09^{e}	1.18 ± 0.02^{d}	0.53 ± 0.01^{b}
LP			
Control	1.29 ± 0.05^{a}	1.29 ± 0.05^{a}	1.18 ± 0.03^{b}
0.1 %	0.83 ± 0.00^{f}	0.83 ± 0.00^{f}	1.99 ± 0.01^{e}
0.2 %	1.09 ± 0.01^{c}	1.09 ± 0.01^{c}	1.58 ± 0.00^{d}

The data are mean values ± standard error. $^{a-f}$ values without the same superscripts within each column differ significantly ($P < 0.05$) PPX, pyrogallol peroxidase; GPX, guaiacol peroxidase; LP, lipid peroxidation

The highest level of MDA was observed in roots of bromus plants treated with the lower concentration of *T. serpyllum* aqueous extract, 24h after the treatment (Table 1). In the bromus leaves, a significant increase in the LP intensity was recorded in plants treated with the lower concentration of *T. serpyllum* aqueous extract, 120 h after the treatment (Table 2).

DISCUSION

One of the effects of allelochemicals on target plants is excess production of reactive oxygen species (ROS) (Inzé and Montagu, 1995; Gniazdowska and Bogatek, 2005). Reactive oxygen species are very toxic to cells and cell can reduce the impact of ROS either by an endogenous system implicating enzymes (Tosun et al, 2009). An imbalance between the formation and inactivation of these species caused oxidative damage to macromolecules namely lipids and proteins (Rani et al, 2004). Allelochemicals might directly inhibit oxidizing enzymes in some way, leaving the plant vulnerable to oxidative damage (Qian et al., 2009).

Study by Zhang (2009) on allelopathy of *T. serpyllum* on weeds shown that water extract of *T. serpyllum* promoted test plants' growth at low concentration but inhibited them at high, and inhibition became stronger as concentration increased. Our research on allelopathy of *T. serpyllum* on soybean plants shown that low concentration of *T. serpyllum* aqueous extract did not induce lipid peroxidation in soybean plants, while the highest concentration used (0.2%) enhanced lipid peroxidation process 72 h after the treatment (Šućur et al., 2013).

In present study lower concentration had greater effects on bromus antioxidant properties than higher concentration. Roots of bromus plants were more affected by allelochemicals than leaves. Many studies have found that roots are more sensitive to allelochemicals than aerial parts of plants (Gatti et al., 2010). An increase in the peroxidases activity and increase in LP intensity in the roots of bromus plants in the first 24 h, probably occurs in response to stress conditions (Sunmonu and Van Staden, 2014). For various plant species a significant increase of lipid peroxidation is observed under oxidative stress. Intensity of LP, and therefore production of malondialdehyde (MDA), exhibit downward trend with duration of the experiment. This could point to the fact that alelopathy provoked stress was not strong enough and scavenging effects of enzyme's could prevent oxidative burst and induction of LP.

CONCLUSION

In conclusion, our results showed that both tested concentrations of *Thymus serpyllum* L. aqueous extract induce lipid peroxidation in bromus plants, but with downward trend with duration of the experiment. Furthermore, concentration of 0.1% of *T. serpyllum* aqueous extract provoked an increase in the peroxidases activity 24h after the treatment. We therefore conclude that tested extract concentrations did not exhibit phytotoxic effect on the bromus. We need more research about this subject to get more information.

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EFFECTS OF BIO-FERTILIZER (*Azotobacter* spp., *Mycorrhiza* spp., *Bacillus spp.*) AND DIFFERENT NITROGEN LEVELS ON FRESH EAR YIELD AND YIELD COMPONENTS OF SWEET CORN (*Zea mays saccharata* Sturt.)

İlknur Akgün[1*] and Cemil Siyah[1]

[1]Suleyman Demirel University, Faculty of Agriculture, Department of Field Crops, ISPARTA- TURKEY, 32260
**Corresponding author: ilknurakgun@sdu.edu.tr*

ABSTRACT

This research was carried out at the experimental area of the Faculty of Agriculture, Suleyman Demirel University, Isparta, Turkey, during 2012 growing seasons. The hybrid variety Merit was used as sweet corn cultivar. A factorial experiment was conducted based on split-plot design in a randomized completed block with three replications. The factors consisted of three doses of nitrogen fertilizer (0, 100, 200 kg N/ha) and bio-fertilizer (*Azotobacter, Mycorrhiza, Bacillus*). The nitrogen fertilizer applications were placed in main plots whereas bio-fertilizer applications were placed in sub-plots. In each block placed 12 plots (0 kgN/ha, 0 + *Mycorrhiza*, 0+*Azotobacter*, 0+*Bacillus*; 100 kgN/ha, 100+*Mycorrhiza*, 100+*Azotobacter*, 100+*Bacillus*; 200 kgN/ha, 200+*Mycorrhiza*, 200 + *Azotobacter*, 200+*Bacillus*). Each plot consisted of 4 lines with 4 meter length, 70 cm row spacing and 20 cm plant spacing. In order to determine agronomic traits (plant height, ear diameter, seed number in ear, number of ears in each plant, fresh ear yield, protein content and total sugar content) 10 plants were randomly selected in each plot. The results showed that nitrogen rates had significant effect on yield, yield components, crude protein content and total sugar content. Significant increase was observed in some characters with applying bio-fertilizers and increasing nitrogen from zero to 200 kg N/ha and but significant differences between 100 kg N/ha to 200 kg N/ha were not observed in most of traits. There was significant interaction between nitrogen doses and bio-fertilizers. The highest fresh ear yield and seed number in ear were obtained by applying 100 kg N/ha with *Azotobacter*. Also the highest protein and total sugar contents were obtained by applying 100 kg N/ha with *Azotobacter* and *Mycorrhiza*. According to results of this study, it can be concluded that applying nitrogen with bio-fertilizers can be reduced amount of nitrogen fertilizer.

Keywords: Sweet corn, bio-fertilizer, nitrogen, agronomic traits

INTRODUCTION

Sweet corn (*Zea mays saccharata* Sturt.) is grown for fresh consumption and canned food industry. Although fresh sweet corn has soft kernels, high sugar concentration, it can be the source of some vitamins (C and E) and minerals (Warman and Havard, 1998). It contains 3.35 g protein, 10 g oil, 221 g carbohydrates, 0.03 g calcium, 1.11 g phospor, 2.8 g potassium per kg (Çetinkol, 1989). It has been reported that the degree of its starch digestion is higher and liquidized kernels of sweet corn are often used for baby food (Kwabiah, 2004).

Grower interest in sweet corn production is increasing year by year in Turkey, especially, Aegean, the Marmara and Mediterranean regions (Turgut, 2000).

Intensive farming practices that aim to produce higher yield, require extensive use of chemical fertilizers. Such product pose a health hazard and microbial population problem in soil, besides making the production cost high (Badran and Safwat, 2004) Many attempts have been tried to replace a part of those harmful fertilizers by bio-fertilizers in maize to get yield of a good quality without loss in its quantity (Zahir et al., 1998, El-Kholy, et al., 2005, Atar et al., 2012). Inoculation of maize seeds with *Azotobacter* and *Azospirillum* increased plant growth, nutrients uptake and yield (Dobbelaere et al., 2001). Furthermore, it has been reported that the recommended dose of chemical fertilizers in corn could reduce using bio-fertilizers without significant yield loss

In Turkey, the producer has applying inorganic N at more than 200 kg ha^{-1} in a season on corn (Kara, 2011). The objective of this work was to examine the effects of seed inoculation with bio-fertilizer (*Micorrhiza, Bacillus* and *Azotobacter* and nitrogen fertilization (0, 100, 200 kg N/ha) on growth and yield of sweet corn grown in Isparta. In addition to the present study, it was to evaluate whether dose of nitrogen fertilization could reduce or not reduce.

MATERIAL AND METHODS

This research was carried out at the Experimental Area of the Faculty of Agriculture, Suleyman Demirel University, Isparta, Turkey during 2012 growing seasons. The seed of Merit F1 was used as sweet corn cultivar. A factorial experiment was conducted based on split-plot design in a randomized completed block with three replications. The factors consisted of three doses of nitrogen fertilizer (0, 100, 200 kg N/ha) and bio-fertilizer (non-inoculation (*Azotobacter*, *Mycorrhiza*, *Bacillus*). The nitrogen fertilizer applications were placed in main plots whereas bio-fertilizer applications were placed in sub-plots. In each block, 12 plots were placed (0 kgN/ha, 0 + *Mycorrhiza*, 0+*Azotobacter ,* 0+*Bacillus;* 100 kg N/ha, 100+*Mycorrhiza*, 100+*Azotobacter,* 100+*Bacillus;* 200 kg N/ha, 200+*Mycorrhiza*, 200 + *Azotobacter,* 200+*Bacillus).* Each plot consisted of 4 lines with 4 meter length, 70 cm row spacing and 20 cm between plants. Seeds were inoculated according to the principles of application. Drip irrigation was applied regularly from seed sowing to harvest. Fertilizer application was made in the spring. The half of N fertilizer as ammonium nitrate and all of P as triple super phosphate (80 kg P_2O_5/ha) was applied with sowing. The other half of nitrogen was applied 25-30 cm plant height manually. In the present study, in order to determine agronomic traits (plant height, ear diameter, seed number in ear, number of ears in each plant, fresh ear yield (de-husked), protein content and total sugar content) 10 plants were randomly selected in each plot.

Sweet corn was harvested at the end of the milking stage when consumed as fresh, five ears of selected 10 plants from each plot were selected randomly, and cobs were harvested. The cobs were immediately frozen by liquid nitrogen to prevent changing from sugar to starch. Sweet corns were analyzed for protein-N x 6.25 (by using Kjeldahl method) and total sugar (Cemeroğlu, 1992) using standard methods.

Data analysis was done according to the methods described using the SAS Statistical Package Program and means between treatments were compared by Duncan Multiple Range Test ($p \leq 0.01$ or $p \leq 0.05$).

Climatic data of the experimental area

Isparta Province has 37° 45' N latitude, 30° 33' E longitude and 1050 m altitude. Isparta has features semi-arid climatic characteristics in the Southwestern Anatolia region. The vegetative periods (from May to August) in 2012 had average temperatures of 21,06 °C and total precipitation of 126,8 mm. The vegetative periods (from May to August) in 2009, 2010 and 2011 had average temperatures of 18.6, 19,6 and 20,9°C, total precipitation of 111,6, 126,1 and 107,7 mm respectively (Anonymous, 2009-2012).. Meteorological data of maize growing seasons was nearly similar compared to the previous there-year average meteorological data.

RESULTS AND DISCUSSION

Significant effects of N level, bio-fertilizer applications and some of their interactions were observed for the some agronomic and quality traits in this study. Statistical analysis of data indicated that N level had significant effects on plant height, number of ears in each plant, ear diameter, seed number in ear, fresh ear yield (de-husked), protein content and total sugar content (Table 1). In generally, while the highest dates were obtained from 100 kg/ha nitrogen dose, the lowest dates were taken at N0 dose. The values of examined characters were increased with applying nitrogen fertilizer. However there was no significant difference between 100 and 200 kg/ha (except for fresh ear yield).

Table 1. The effect of nitrogen doses and bio-fertilizer (*Azotobacter*, *Mycorrhiza*, *Bacillus*) on the some agronomic and quality traits in the sweet corn

Treatment	Examined Traits						
	Plant height (cm)	Ears number/plant	Ear diameter (cm)	Seed number/ear	Fresh ear yield (g/plant)	Protein content (%)	Total sugar (mg/100g)
Nitrogen							
N_0	180,33b	1,25b	4,85b	644,67b	350,65c	13,17b	12,72b
N_{100}	193,26a	1,49a	5,00a	705,82a	452,97a	14,26a	13,29a
N_{200}	187,73a	1,48a	5,03a	699,87a	386,73b	13,46ab	13,29a
Bio-fertilizer							
Bacillus	187,67	1,44ab	5,00ab	692,09ab	388,87b	13,64	12,72
Azotobacter	185,77	1,52a	5,08a	720,05a	413,87a	13,57	13,29
Mycorrhiza	189,64	1,35bc	4,95b	674,62bc	400,47ab	14,10	13,29
Non- inoculation	185,33	1,31c	4,81c	646,58c	383,93 b	13,12	13,06
F_N	16,88**	22,87**	7,72**	25,93**	69,36**	6,41**	25,29**
$F_{\text{bio-fertilizer}}$	1,17	8,95**	7,95**	3,83*	3,43*	2,24	2,64
$F_{\text{Nx bio-fertilizer}}$	7,54**	4,67**	12,68**	12,83**	1,81	17,32**	1,55
CV (%)	2,92	6,69	2,50	3,35	5,44	5,89	3,77

** and * Significant difference at %1 and %5 levels, respectively

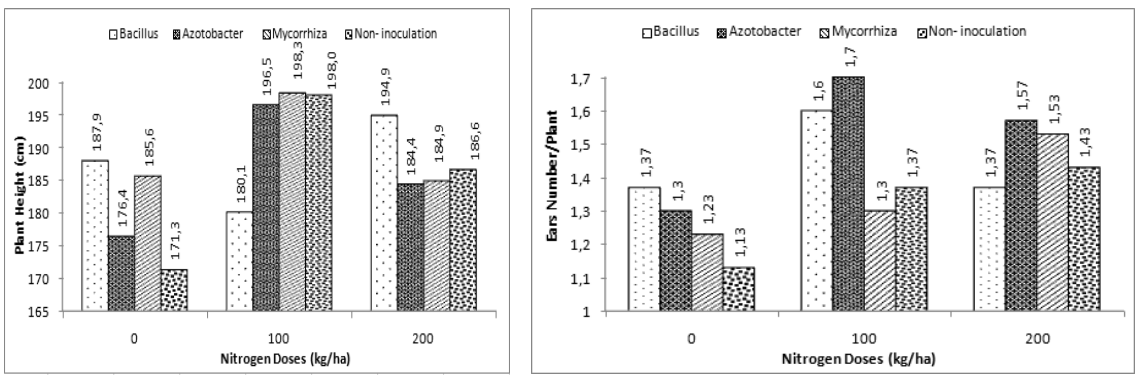


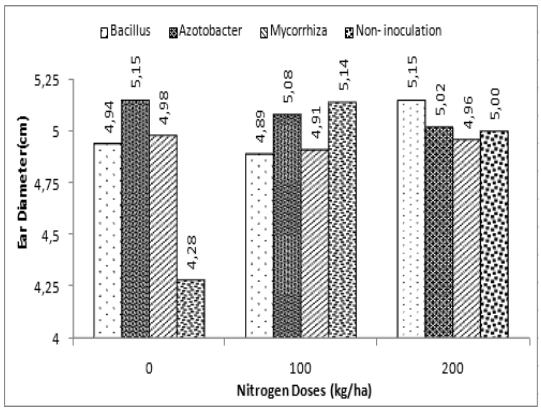
Figure 1. The effect of Nitrogen doses and bio-fertilizer (*Azotobacter*, *Mycorrhiza*, *Bacillus*) on plant height and numbers of ears in each plant in the sweet corn

Effect of the bio-fertilizers (*Azotobacter, Mycorrhiza*, *Bacillus*) and nitrogen applications on some agronomic and quality traits can be seen on Table 1. It is clearly said that examined traits were positively affected by the bio-fertilizers applications. It was found that the differences between the bio-fertilizers applications were statistically significant. In general, while the highest values in the examined characters were obtained from especially *Azotobacter* applications, the lowest dates (without total sugar content) were taken from non inoculation ones. Similar results were found in various species by Sing et al., (2004) and Naseri and Mirzaei, (2010).

Microorganisms can improve crops yield by synthesize plant growth hormones (such as IAA and GA) or making nutrients available for uptake by plant (Dobbelaere et al., 2001; Senthil-Kumar et al., 2009).

Results obtained from analysis of variance for plant height indicated that there were not statistically significant differences between the effects of the bio-fertilizers, but interaction both of them (Nxbio-fertilizer) was statistically significant. The lowest plant height was observed with non-inoculation at N0 level. Effects of *Azotobacter and Mycorrhiza* with using nitrogen fertilizer (100 kg/ha) increased (Fig. 1).

The number of ears in each plant was increased with applying bio-fertilizer at N0. On the other hand, effects of bio-fertilizers changed with using nitrogen fertilizer (Fig. 1). The highest value was obtained from especially *Azotobacter* applications.



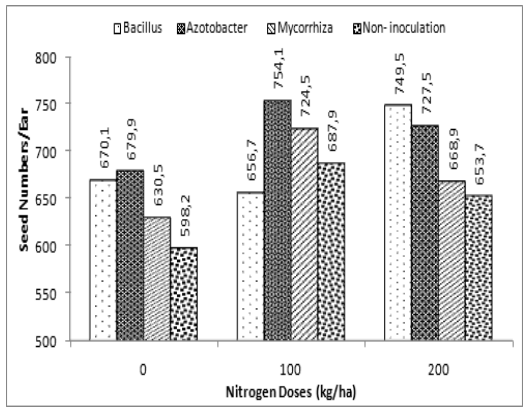
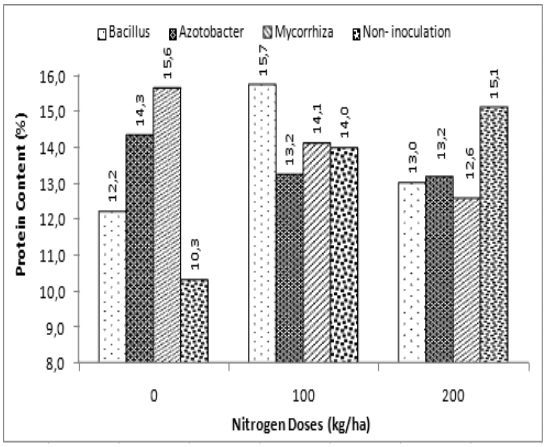


Figure 2. The effect of Nitrogen doses and bio-fertilizer (*Azotobacter*, *Mycorrhiza*, *Bacillus*) on ear diameter in each plant and seed numbers per ear in the sweet corn



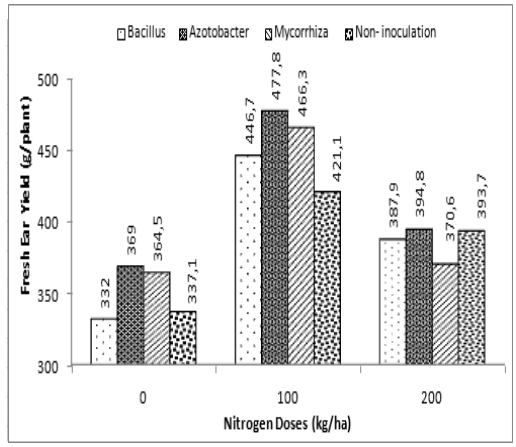


Figure 3. The effect of Nitrogen doses and bio-fertilizer (*Azotobacter*, *Mycorrhiza*, *Bacillus*) on fresh ear yield (g/plant) and protein content in the sweet corn

Ear diameter was influenced by nitrogen fertilizer and bio-fertilizer treatment and their interaction (Table 1). This trait was increased by increasing nitrogen levels. While all kinds of bio-fertilizer had similar effects on this trait at non-nitrogen application, with using nitrogen fertilizer effects of them were changed (Fig 2).

The seed numbers per ear showed an increase by nitrogen fertilizer amount, but between 100 and 200 kg/ha was not determined a significant difference. The seeds per ear increased due to inoculation in comparison with non-inoculation (Fig. 3)

The analysis of variance indicated that there are significant differences between nitrogen fertilizer, bio-fertilizer and their interaction on fresh ear yield (g/plant) in the sweet corn. The lowest fresh ear yield was recorded non nitrogen fertilizer treatment. The fresh ear yield were increased by using nitrogen fertilizer, but in the highest dose (200 kg/ha) it decreased and statistical significant difference was determined between 100 and 200 kg/ha. The highest fresh ear yield due to bio-fertilizers was followed by nitrogen fertilizer. However, effects of bio fertilizers were decreased at high nitrogen level (Fig.3).

The analysis of variance showed that protein and sugar content were affected significantly by nitrogen fertilizer, but not affected by bio-fertilizer (Table 1). Also, their interaction effects on protein contents were statistically significant. The lowest protein content was obtained from non nitrogen fertilizer and bio-fertilizer treatments. Protein content increased with inoculation treatments at N0 level (Fig. 3).

Although total sugar was not affected by inoculation, total sugar was different after nitrogen application with bio-fertilizer and total sugar was increased (Fig. 4). According to general means, it was found that the lowest total sugar content was in *Bacillus* application (12,72 mg/100g), but the difference was not statistically significant (Table 1).

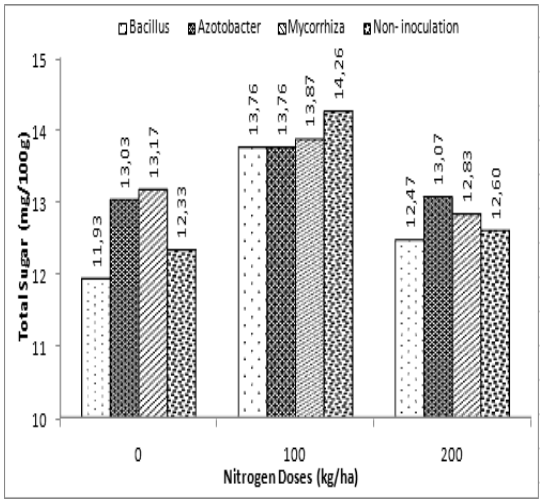


Figure 4. The effect of Nitrogen doses and bio-fertilizer (*Azotobacter*, *Mycorrhiza*, *Bacillus*) on total sugar content in the sweet corn

CONCLUSION

Our results clearly indicated the beneficial effect of bio-fertilizer in sweet corn. Combined inoculations with nitrogen fertilization had a strong positive effect on examined characters in sweet corn. On the other hand, when nitrogen doses were increased, effect of bio-fertilizer was decreased. According to the results, it can be concluded that applying the combined bio-fertilizer and chemical nitrogen fertilizer can be helpful method to increase yield in sweet corn and reduce environmental pollutions.

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INTEGRATED PROTECTION IN FORESTRY AND LANDSCAPE ARCHITECTURE

ИНТЕГРИРОВАННАЯ ЗАЩИТА В ЛЕСНОМ ХОЗЯЙСТВЕ И ПЕЙЗАЖНОЙ АРХИТЕКТУРЕ

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Plant Protection Society of Serbia, IOBC-EPRS, IOBC-WPRS, Belgrade, 2015, pp. 203 - 205

ИНТЕГРИРОВАННАЯ ЗАЩИТА ДУБРАВ ОТ ВРЕДНЫХ НАСЕКОМЫХ В РОССИИ

Николай Иванович Лямцев

Всероссийский научно-исследовательский институт лесоводства и механизации, Пушкино, Московская обл., Россия
lyamtsev@vniilm.ru

Обеспечение воспроизводства дубрав не возможно без интеграции лесохозяйственных и лесозащитных мероприятий и перехода к стратегии управления популяциями насекомых-вредителей (Знаменский, 1987).

С целью защиты дубрав от хозяйственно опасных насекомых необходимо осуществлять: 1) лесопатологический мониторинг и обследования; 2) прогнозирование изменения численности насекомых, угрозы повреждения насаждений, ожидаемого ущерба и планирование работ; 3) профилактические и специальные защитные мероприятия; 4) оценку их эффективности.

Лесопатологический мониторинг и обследования

Для эффективного проведения защитных мероприятий необходимо своевременно выявлять начало массового размножения насекомых, определять заселенные участки и границы очагов, плотность и состояние популяции, фазу градации и результативность их естественных врагов. Оценка качественных и количественных параметров популяций насекомых и состояния (прежде всего дефолиации) насаждений проводится выборочным методом. Для этого осуществляются долговременные стационарные наблюдения, а в случае обнаружения повреждения насаждений и роста численности насекомых – лесопатологические обследования.

Учет численности листогрызущих вредителей дубрав в зависимости от их вида осуществляется в кроне дерева, на стволах, в подстилке и верхнем слое почвы. Разработаны последовательные планы учетов кладок яиц, гусениц и куколок насекомых. Остается актуальной задача разработки системы учета насекомых на больших территориях для оптимизации сети лесопатологического мониторинга.

Выявление очагов листогрызущих вредителей дубрав осуществляется преимущественно наземными способами. Дистанционные методы разработаны недостаточно.

Методы феромонного мониторинга листогрызущих вредителей дубрав разработаны достаточно детально, но практическое их применение невелико. Необходимо расширять использование феромонных ловушек особенно для выявления возникающих очагов, что очень важно для своевременного проведения лесозащитных мероприятий, например избирательных локальных истребительных обработок.

Лесозащитное прогнозирование

Основой для планирования активных лесозащитных мероприятий служит краткосрочный прогноз дефолиации насаждений (Лямцев, 2004). Он осуществляется: 1) по таблице критических чисел – экспериментальным оценкам плотности популяции насекомых, при которой наиболее вероятно полное объедание насаждений; 2) по модели, используя оценки выживаемости гусениц, их лабораторные кормовые нормы и численность на единицу количества корма (100 г листьев); 3) по регрессионным уравнениям (зависимости дефолиации от численности насекомых).

Для наиболее опасных насекомых составлены модели оценки потерь прироста и гибели деревьев после повреждений разной интенсивности (степени и продолжительности (кратности) дефолиации).

Составлены алгоритмы для принятия решений о необходимости применения инсектицидов. Определены экономические пороги вредоносности – уровни численности насекомых или дефолиации, при которых целесообразны лесозащитные мероприятия. При такой численности угроза объедания дубрав составляет 50% и выше.

Пороги вредоносности установлены для непарного шелкопряда (*Lymantria dispar* L.), зеленой дубовой листовертки (*Tortrix viridana* L.), златогузки (*Euproctis chrysorrhoea* L.), зимней пяденицы (*Operophthera brumata* L.), боярышниковой листовертки (*Archips crataegana* Hbn.), пядениц-шелкопрядов. Работы по созданию системы принятия решений и планирования применения инсектицидов на основе эколого-экономических критериев необходимо продолжить.

Разработка и применение различных средств и методов ограничения численности листогрызущих вредителей

Обработка крупных очагов наиболее эффективна способом авиационного ультрамалообъемного опрыскивания. Химические инсектициды обеспечивают высокий защитный эффект, но снижают численность полезных насекомых, что способствует более быстрому восстановлению популяции вредных насекомых и переходу во вспышечное состояние. Бактериальные препараты не оказывают прямого отрицательного влияния на нецелевые объекты. Наблюдается снижение эффективности паразитов в связи с их гибелью на ранних стадиях развития вместе с насекомыми-хозяевами. Применение новых препаративных форм микробных инсектицидов способом ультрамалообъемного опрыскивания благодаря образованию более мелких капель (50-200 микрон) снижает норму расхода препарата до 3 л/га и обеспечивает высокую (85% и более) эффективность. Степень повреждения листвы после обработки насаждений должна быть менее 30 %.

Одной из важных проблем развития биологического метода защиты леса является разработка стратегии и тактики его применения. Пока в основном происходит лишь замена химических пестицидов на микробиологические. Однако биологический метод во многом должен иметь профилактическую направленность.

Важным моментом интеграции лесозащитных мероприятий является оптимизация сочетания действия химических и микробиологических инсектицидов, а также природных энтомофагов и патогенов вредных насекомых. Основной путь сохранения энтомофагов при применении инсектицидов – это выбор более безопасных для полезной энтомофауны сроков обработки (по гусеницам I-II возрастов). Большое значение имеет также избирательный характер защиты насаждений и проведение работ в год, предшествующий фазе кульминации вспышки массового размножения, с целью предотвращения сильной дефолиации, а не полного уничтожения вредителей (Знаменский и др., 1976; Знаменский, 1987).

Наиболее перспективна интеграция энтомофагов с применением бактериальных и вирусных препаратов. Инфицирование листогрызущих насекомых стимулирует развитие других заболеваний, повышенную смертность гусениц старшего возраста и куколок, снижение плодовитости (Знаменский и др., 1976).

Применение препаратов по типу биологического инсектицида является в настоящее время основным и позволяет получить более быстрый и надежный эффект (Штерншис и др., 2000). Используются бактериальные препараты на основе Bacillus thuringiensis (Bt). Выбор конкретного препарата обусловлен неодинаковой восприимчивостью разных видов насекомых к одному и тому же препарату. Большое значение имеет состав входящих в него спор и токсинов.

Наиболее многочисленна группа препаратов, содержащих споры и кристаллы бактерий. Вторая группа препаратов, кроме спор и кристаллов, содержит термостабильный β-экзотоксин. В качестве действующего начала в бактериальных препаратах могут быть также только очищенные токсины, вырабатываемые бактерией. Содержание экзотоксина расширяет сферу и повышает биологическую эффективность применения препаратов за счет иного механизма действия экзотоксина по сравнению с эндотоксином. Он может действовать не только через кишечник, но и через покровы насекомых, а в комбинации со споро-кристаллическим комплексом проявляет синергизм (Штерншис и др., 2000). Поэтому при выборе и применении биопрепаратов следует обращать внимание на природу действующего начала. Если это микробный метаболит, то действие препарата менее зависит от экологических факторов внешней среды, чем действие препарата на основе спор или клеток микроорганизма.

Разработка таких технологий требует изучения взаимодействия различных групп естественных врагов вредителей в лесных экосистемах, выявления возможности синергизма микробиологических

инсектицидов и важнейших паразитов листогрызущих насекомых (Лямцев, 2003, 2005). Нами установлено, что для дубрав, особенно важным является повышение биологической устойчивости насаждений, видового разнообразия и численности энтомофагов в результате применения лесохозяйственных и специальных профилактических мероприятий.

Без осуществления таких мероприятий невозможно эффективное применения микробных препаратов против насекомых, быстро восстанавливающих высокую численность и образующих хронические очаги (например, зеленой дубовой листовертки). Если эффективность биоценотических регуляторов (энтомофагов) низкая, то и при применении бактериальных препаратов возможно восстановление высокой численности вредных насекомых. «Бумеранг-эффект» наблюдался нами в дубравах Саратовской обл. после обработки очагов зеленой дубовой листовертки бактериальным препаратом лепидоцидом.

При планировании истребительных мероприятий против насекомых, имеющих более эффективный комплекс энтомофагов (например, непарного шелкопряда) для защиты насаждений достаточно применения инсектицидов с периодичностью раз в 10 лет. Основным условием получения положительного результата является наличие в насаждении определенного запаса естественных врагов (зараженность паразитами гусениц и куколок не менее 20%) и обработка на 3-4 год после начала подъема численности вредителей.

За последние 10 лет не произошло существенного сокращения доли биологических препаратов в общем объеме средств защиты леса. Высокая доля микробиологических препаратов обусловлена значительной площадью очагов, в которых запрещено применение химических пестицидов, а также наличием в лесных экосистемах достаточно эффективных популяций паразитов и хищников, которые компенсируют менее высокую (по сравнению с химическими препаратами) эффективность биологических инсектицидов.

Негативным является значительное сокращение ассортимента применяемых для защиты лесов препаратов. В Государственном каталоге пестицидов и агрохимикатов, разрешенных к применению на территории Российской Федерации в 2014 г., было только семь препаратов для защиты лесов авиационным способом: четыре биологических (лепидоцид, СК; лепидобактоцид, Ж; битоксибациллин, П; битиплекс, СП), димилин, СП, фьюри, ВЭ и таран, ВЭ.

Для обеспечения защиты лесов необходимо расширение ассортимента препаратов с учетом особенностей конкретных видов вредителей и лесопатологической ситуации. Требуется продолжение исследований с целью повышения качества инсектицидных препаратов и технологий их применения, выявления побочного действия на нецелевые объекты - полезную лесную энтомофауну (паразитических и хищных насекомых). Необходим постоянный поиск активных штаммов энтомопатогенных микроорганизмов, улучшение рецептурной формулы создаваемых препаратов, совершенствование наземной опрыскивающей аппаратуры.

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ЛЕСОПАТОЛОГИЧЕСКОЕ СОСТОЯНИЕ ГОРНЫХ ЛЕСОВ КАЗАХСТАНА

Абай Сагитов, Нуржан Мухамадиев и Нурсагим Ашикбаев

Казахский НИИ Защиты и карантина растений
г. Алматы, Казахстан, nurzhan-80 mail.ru

АБСТРАКТ

В статье приведены результаты лесопатологического мониторинга горных лесов Казахстана. Установлена категория состояние деревьев и основные вредители и болезни. Определена численность ксилофагов на феромоновых ловушках. Отмечены три новых видов ксилофагов в условиях Заилийского Алатау. Испытаны перспективные инсектициды и биопрепараты против листогызущих вредителей.

Ключевые слова: Лес, Вредитель, Фитопатоген, Лесозащита.

ВВЕДЕНИЕ

В одном из ранних Послании Президента страны народу Казахстана (10 октября 1997 года) говорится: «Символом нашей страны в будущем должны быть не пустыни, а леса». Это должно быть программой действия нынешнего и последующих поколений лесоводов в XXI веке. Лесистость республики составляет лишь 4,5% территории, покрытая лесом угодья занимают 12,3 млн. га. Их исчезновению угрожают не только отрицательные антропогенные факторы, но и некоторые природные биологические факторы, например, эпифитотии болезней или вспышка массового размножения вредителей. Иногда нашествие опасных вредителей ставит под угрозу существование лесных массивов своевременная защита от насекомых-вредителей, следует считать весьма актуальной задачей [Коваль И.А. 2007г., Сагитов А.О. 2003 г.]. Цель исследования уточнить лесопатологическое состояние лесов в горно-таёжной части Восточного региона и Заилийского Алатау.

МАТЕРИАЛЫ И МЕТОДЫ

Общепринятые методы в лесопатологических обследований и лесной энтомологий. Для выявления мест зимовок хвое - и листогрызущих вредителей рано весной и осенью в лесу осматривают в среднем 10 модельных деревьев (их площадки под кронами), с признаками повреждения (шелкопряд, боярышница и др.). Закладывают пробы размером 50х50 см под кронами деревьев в районе проекции их крон с примыканием узкой стороны пробы к стволу. Лесную подстилку и верхние слои почвы просматривают до 20 см, в зависимости от типа почв, обнаруженные куколки помещают в банку или коробку с этикетками с указанием лесничества, № квартала и даты учета. В лаборатории подсчитывают количество живых и пораженных болезнями и паразитами куколок. Отобранных здоровых куколок распределяют на самцов и самок, устанавливают соотношение полов и взвешивают. Куколок помещают в садки для дальнейшего наблюдения.

Оценка интенсивности объедания насаждений производят на основании соответствующей шкалы. С этой целью различают 4-градации повреждения древостоев:

- Сплошное - до 75-100%;
- Сильное - от 50-75%;
- Среднее – от 25-50%;
- Слабое – до 25%.

Динамика болезней изучается путём регулярных учетов и наблюдений при маршрутных обследованиях и стационарно на определенном участке в течение всего вегетационного периода. Учеты проводятся по диагонали поля через каждые 10 дней,

по 10 деревьев в 10 точках. При этом устанавливаются сроки первоначального проявления болезней и дальнейшее их развитие, проводятся учеты, распространения и степень развития болезней по общепринятым методикам.

Распространение болезней определяют по проценту больных растений от общего количества учетных растений и рассчитывают по формуле:

$$P = \frac{nx100}{N}, \text{ где}$$

P - распространенность болезни, (%);

n - количество больных растений при учете;

N - количество учетных растений.

Степень развития болезни учитывается по шкале:

0 - отсутствие поражения

1 балл - поражено до 10% листовой поверхности (листа, растения)

2 балла - поражено от 11 до 25 % поверхности (листа, растения)

3 балла - поражено от 26 до 50 % поверхности (листа, растения)

4 балла - поражено свыше 50% поверхности (листа, растения)

Стволовые вредители, главном образом короеды и их показатели размножения учитывают на круговых палетках длиной 50 см (для мелких короедов - 30 см, для усачей - 1 м) закладываемых в середине средних по размером заселенных стволов, остатков и. д [Ильинский 1965,1975 г.]. Для определения координат мест нахождений мониторинговых площадок будет использован прибор GPS.

РЕЗУЛЬТАТЫ

В лесах Рудного Алтая в пихтовых насаждениях на 6 лесничествах было заложена пробная площадь в количестве 100–150 деревьев, для оценки лесопатологического состояние, где установлено усыхающих деревьев составило 12,5–35,0%. Причиной тому являются фитопатогенные грибы корневая губка, ржавчина хвои и размножения вредных сосущих насекомых и стволовых вредителей: тли, ложнощитовки, короеды, усачи. В целом лесопатологическое состояние лесов Рудного Алтая оценивается как ослабленное.

Ржавчина хвои на пихте встречаются по все местно, развитие болезни составляет более 56,6%, корневая губка составляет 18,9–34,8%. Распространенность ржавчины и корневой губки была высокой 98,7 и 65,5% соответственно, что связано с условиями произрастания и увлажненным климатом.

Доминирующими вредителями в лесах являются боярышница, березовая пяденица и осиновая узорчатая моль в наибольшем количестве встречаются в ГУ: «Катон – Карагайский ГНПП», «Зыряновский ЛХ», «Риддерский ЛХ», «Верх – Убинский ЛХ», «Черемшанский ЛХ» старшего класса возраста, средней полноты 0,5–0,6. В среднем на 1 дерево численность вредителей составляет – 89 шт. гусениц боярышницы, 14 шт. – гусениц березовой пяденицы, где вредоносность боярышницы составляет – 100%, березовой пяденицы – 25%, узорчатой моли - 95%. В последние годы основной причиной ослабления древостоев стало поражение древостоев корневыми гнилями (губкой корневой – 35% от общей площади насаждений ослабленных под воздействием болезней), стволовыми гнилями (осиновый, ложный, окаймленный, еловый, настоящий трутовики – 32%) и некрозно – раковыми заболеваниями (рак серянка – 21%).

В очаге боярышницы встречался активный хищный энтомофаг жужелица *Carabus alpestris* Sturm. Численность в очагах боярышницы в среднем на 1 га составляло 11–13 экземпляров. Из паразитов на гусенице боярышнице, пяденицы, шелкопряда встречались *Microgaster* sp., и *Bracon* sp. где паразитированность ими составляло 4–8%. Также в очаге боярышницы встречались погибшие гусеницы от бактериоза 8–16%.

Испытанные препараты против гусениц боярышницы показали высокую биологическую эффективность. На 7–ой день учета биологическая эффективность препаратов, димирон, и биопрепарат ак көбелек составили 77,2 и 82,5%, на 14–ый день составили 87,5–89,0%. Таким образом, по результатом испытании препаратов можно судить, что против хвое и листогрызущих вредителей лесов Рудного Алтая есть достаточно эффективные биопрепараты и инсектициды 4–го класса, опасности которых можно будет применять при увеличении численности вредителей.

По результатам НИР подготовлены рекомендации по системе защитных мероприятий от основных хвое и листогрызущих вредителей в лесах Рудного Алтая Восточно-Казахстанской области.

17 мая 2011 года прошедший ветровал в государственном природном парке «Медеу» и Иле-Алатауском государственном национальном природном парке в ущелье Медео на общей площади 480 га в объеме 96 тысяч кубометров. Нами при обследовании установлена численность, и заселенность короедами поваленных деревьев увеличивается и переходят на рядом стоящие деревья.

Средняя заселенность стволовыми вредителями *(Ips hauseri* Reitt., *Pityogenes* sp., и др.*)* в местах складирования на одну палетку 1дм^2 древесины составило от 2 до 6 шт., а на 1,5 м древесины колебятся

в пределах от 21±6,5 до 60,8±6,9 шт. стволовых вредителей. При данной плотности поселения стволовых вредителей прогнозируется увеличение численности в последующие периоды.

В урочище Медеу Мало-Алматинском и Бутаковском лесничествах на прилегающей территории ветровала (Горельник и участок № 4) насаждения 3-4 класса возраста тянь-шаньской ели заложена пробная площадь (в количестве 100 деревьев), для оценки категории состоянии деревьев таблица 1.

Из таблицы 1 видно, что отпад деревьев в среднем составил 27 - 29 %. Растущие на корню деревья подверглись нападению насекомых-ксилофагов: короеды, усачи, златки, долгоносики и др. В целом лесопатологическое состояние леса неудовлетворительное (рисунок 1), что вызывает определенную угрозу оставшимся рядом растущим деревьям, так как они уже подвергнуты повторному массовому нападению стволовыми вредителями.

Основная площадь очагов вредителей представляют насаждения, заселенные стволовыми вредителями. Поврежденные площади вторичными вредителями - это ветровальные деревья и их остатки (пни, ветки, стволы) которые находятся в основном в труднодоступной горной зоне рисунок 2.

Таблица 1. Результаты оценки лесопатологического состояния деревьев на пробных площадях

Место закладки мониторинговых площадок	Категория состояния деревьев, %				Сохранилось деревьев, %	Общее количество деревьев, шт.	Отпад деревьев, %
	I	II	III	IV			
Медеу (плотина) H - 2200 N - $43^0$09’354 E - $077^0$03’012	3	22	40	5	70	100	29
Бутаковка H - 2082 N - $43^0$10’338 E - $077^0$06’248	-	-	65	7	72	100	27

Рисунок 1. Усыхающие деревья в ущелье Медеу и на прилигающих участках ветровала (2014 г.).

Рисунок 2. Ветровальный участок урочище Медеу

Мониторинг короедов осуществляли с помощью двух типов феромоных ловушек Ипсвабол – Д барьерного и треугольного типа, приобретенные из Белорусии и Молдавии. Лёт жуков отмечен при средней температуре 18^0 С (конец апреля – начала мая).

В результате мониторинга на участках ветровала обнаружено основные виды вредителей: семиреченский еловый дровосек (*Tetropium staudingeri* Pic.), рагий ребристый (*Rhagium inguisitor* Z.), азиатский гравер (*Pityogenes perfossus* Bees.), короед Гаузера (*Ips hauseri* Reitt.), шестизубый короед (*Ips sexdentatus* Boern.), тянь-шаньский рогохвост (*Sirex tianshanicus* Sem.), которые представляют потенциальную опасность в последующие годы ослабленным и здоровым насаждениям ели Заилийского Алатау.

Отловленные на феромонных ловушках вредители были: из семейства короедов *(Ipidae):* древесинник хвойный *(Triptodendron linatium* Ol.), типограф (*Ips typographus* L.), гаузер (*Ips hauseri* Reitt.), короед двойник (*Ips duplicates* Sahalb.), гравер обыкновенный (*Pityogenes chalcographus* L.) Байкальский гравер (*Pityogenes baicalicus* Egg.*)*, киргизский гравер (*Pityophtorus kirgisicus* Pjat.) рисунок 3.

Рисунок 3. Вредители, попадавшие в феромонные ловушки в 2013 году (%)

Шестизубый короед, короед типограф, рагий ребристый ранее не отмечались в условиях Заилийского Алатау. Вероятно, они проникли с зараженным лесом, поступавшим в регион для строительства в 60-70-ые годы прошлого столетия

Обсуждение. Обнаруженные виды ксилофагов ранее не отмечались в горах Заилийского Алатау. Для предотвращения угрозы, нависшей над хвойными лесами Заилийского Алатау и Рудного Алтая, как особо охраняемой природной территории и других хребтов Тянь-Шаня, расположенных на территории страны, основную роль должно сыграть соблюдение правил как внешнего, так и внутреннего карантина, а также разработка проведение комплексных мероприятий по недопущению массового размножения карантинных и опасных вредителей. Важным направлением будет совместные исследования зарубежных ученных по применению перспективных энтомофагов для снижение численности вредных насекомых-вредителей леса.

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Нами были собраны экземпляры жуков на феромоновые ловушки предположительно короеда-типографа. Поэтому для подтверждения видовой идентификации обьекта были переданы в Грузию, видному систематику короедов (зав. отделом «Защиты леса» Института Леса Грузии Тбилиси Арчилу Супаташвили который подтвердил наши предположения, что это жук короед-типограф. За что мы выражаем свою благодарность за определение жука короеда.

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A CONTRIBUTION TO THE KNOWLEDGE OF THE PHYTOPHAGOUS JEWEL BEETLES (*Coleoptera: Buprestidae*) OF THE FRUŠKA GORA NATIONAL PARK

Dejan V. Stojanović[1,2], Srećko B. Ćurčić[3] and Tatjana B. Kereši[4]

[1]Institute of Lowland Forestry and Environment, University of Novi Sad, Antona Čehova 13, 21000 Novi Sad, Serbia
[2]Fruška Gora National Park, Zmajev Trg 1, 21208 Sremska Kamenica, Serbia
[3]Institute of Zoology, University of Belgrade - Faculty of Biology, Studentski Trg 16, 11000 Belgrade, Serbia
[4]University of Novi Sad - Faculty of Agriculture, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia
E-mail: dejanstojanovic021@yahoo.co.uk

ABSTRACT

In the paper we presented the overview of 14 potentially harmful jewel beetle species (Coleoptera: Buprestidae), mostly in forestry, that are inhabiting the Fruška Gora National Park (North Serbia).

For each determined species of jewel beetles the following data are provided: the finding place and date, flight period, host plants and abundance. The data were collected by the Diagnostic and Prognostic Service of the Fruška Gora National Park at many localities during the 10-year research in the national park in order to recognize a possible need to undertake the forest protection measures. Identified species of jewel beetles didn't have such abundance to cause harmful impacts on the host plants in protected forest areas in the Fruška Gora National Park.

Key words: Coleoptera, Buprestidae, harmful jewel beetles, Fruška Gora National Park.

INTRODUCTION

Jewel beetles or buprestids are thermophilous insects which can be found on sunny places warmed by direct sunlight. The body is strong, elongated and oval, with a variety of colors, often possessing noticeably metallic lustre. The majority of species are distributed in the tropics (Muskovits and Hegyessy, 2002).

According to the newest estimations, the world fauna of jewel beetles contains 15,000 species (Muskovits and Hegyessy, 2002). The family Buprestidae is divided into 13 subfamilies, 50 tribes, and more than 500 genera and subgenera (Bellamy, 1985). Around 470 species belong to the European fauna of jewel beetles, while approximately 200 species are known for Central Europe (Chatenet, 2000). German fauna contains approximately 100 Buprestidae species, Bulgarian contains 179 species (Sakalian, 2003), fauna of Serbia comprises 58 species (Ćurčić et al., 2001; Sakalian & Ćurčić, 2001), while Popo (1981) cited 179 species of buprestids for the area of the former Yugoslavia. Totally 44 Buprestidae species from Central Europe are reported as harmful for forest trees (Vasić, 1981).

In the Balkan countries the jewel beetles were scarcely researched, except in Bulgaria (Sakalian, 2003) and Greece (Mühle et al., 2000). The inventarisation of the family Buprestidae is still not comprehensively conducted in Serbia. The faunistic lists are not complete and the role in forest habitats is still not enough known in the country. A high abundance of jewel beetles in forest habitats during the permanent monitoring points to the real importance of this group of insects due to the significant harmfulness caused mostly by the xylophagous larvae (Vasić, 1981; Mihajlović, 2008).

In the Handbook of Reporting and Diagnostic-Prognostic Service of Forest Protection in the former Yugoslavia, within the chapter about harmful insects from the order Coleoptera, it was given a significant attention to the family Buprestidae (Vasić, 1981). In the extensive text, it was highlighted the significance of gradations of some jewel beetles and gradations in combination with other pest insects.

Therefore, the reporting and diagnostic-prognostic services in national parks have to record the dynamics of populations of potentially harmful jewel beetles and to make estimations and forecasts of the abundance. Thus, potentially increasing number of specimens of pest jewel beetles would lead to performing on-time prevention measures in order to avoid related damages (Vasić, 1981).

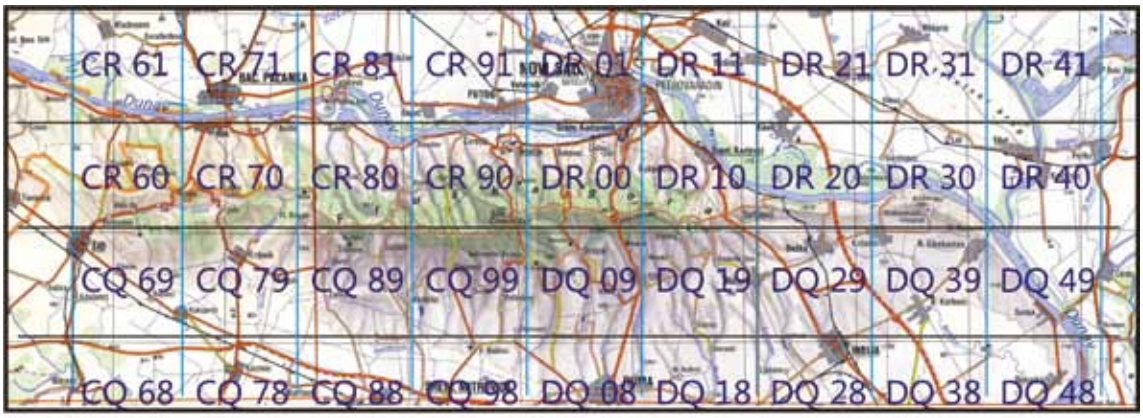
In deciduous forests in the Republic of Serbia it was highlighted the importance of some pest jewel beetles (Mihajlović, 2008). Primary physiological pests in the country are *Coraebus florentinus* (Herbst, 1801) and *Agrilus biguttatus* (Fabricius, 1776). *Ovalisia rutilans* (Fabricius, 1777) is a secondary pest and attacks physiologically weakened trunks, while *Agrilus viridis* Linnaeus, 1758 (attacks beech, oak, linden, birch and alder) and *Chrysobothris affinis* (Fabricius, 1794) (attacks oak and beech) are both primary and secondary pests. In coniferous forests in the Republic of Serbia it was pointed out the importance of the blue pine wood borer *Phaenops cyanea* (Fabricius, 1775) (attacks physiologically weakened and dry pine trees) and *Capnodis tenebrionis* (Linnaeus, 1758) (attacks roots of various fruit trees and can make significant damages). In poplar forests, primary and secondary pest *Trachypteris picta* (Pallas, 1773) may cause serious damages (Jodal, 1963; Mihajlović, 2008).

The aim of the research presented in this paper was to make inventarisation of potentially harmful species of jewel beetles which live in forest habitats of the Fruška Gora National Park (North Serbia). The obtained results may indicate on possibilities for potential outbreaks of the recorded species. Such data are very important for making policy for protection of forest ecosystems in protected areas of the national park from possible gradations of insect pests and related damages. Presented data obtained by permanent monitoring may indicate trends of increasing or decreasing of abundance of some species and, consequently, related harmful effects that may be caused.

In the future, the obtained share of pests in total number of jewel beetle species in the Fruška Gora National Park may be compared with the related numbers in other protected nature areas in the Republic of Serbia, as well as in the neighbour countries.

From the geological perspective, the explored area of the Fruška Gora National Park represents the outermost eastern edge of the Dinarides. It is located on the edge of temperate continental climate, and also takes the characteristics of sub-continental and mountain climate due to the changes of altitudinal gradient and impact of forests (Pešić and Stojanović, 2008).

Mt. Fruška Gora is a forested region, but from the former 130,000 ha only 23,000 ha remain under forests. It is characterized by 33 types of forests; among them, mixed forests of common oak, types of forests with sessile oak and common hornbeam, as well as pure common oak and beech forests prevail. Forests of Turkey oak and pubescent oak prevail in xerothermic habitats. Fifty-four species of trees are recorded there, of which 17 species are introduced. The dominant species are silver lime (37.6%), followed by sessile oak (18.8%), Turkey oak (11.8%), beech (8.8%) and hornbeam (6.6%). Introduced species have a share of 5.9%, of which black locust has a share of 2.1%. Coniferous forests take share of somewhat less than 2% in the forest communities. Steppes have been forced out as a result of anthropogenic influence and only at the margins of the national park are preserved in the guise of fragments of a *Festucion rupicolae* Soó 1940 union. Meadow communities are of secondary origin, arising as a result of forest clearing (Pešić and Stojanović, 2008).



Map 1. UTM squares (10 x 10 km) designated within the confines of the Fruška Gora National Park.

MATERIAL AND METHODS

The research area where the jewel beetle specimens were found is shown on a map with UTM grid (Map 1). Standard methods are used for collecting and monitoring jewel beetles for the research purpose. Jewel beetles were collected by insect net, but also by hand on trunks, branches, leaves, stacks and flowers of different herbaceous or shrub plants.

The adults of jewel beetles were collected during 2003, 2005, 2008, 2009, 2010, 2011, 2013 and 2014 at 21 localities by the first author of the study. For the purpose of this research, the material was collected at the following localities, which can be found on Map 1:

Popovica (DR 00) - elevation of 400 m a.s.l.; meadows at the edge of a forest of sessile oak and Turkey oak; Iriški Venac (DQ 19) - elevation of 450 m a.s.l.; meadows in a sessile oak forest or at the edge; Stražilovo (DR 10) - elevation of 190 m a.s.l.; spacious meadows in a forest of sessile oak and hornbeam with sweet woodruff; Letenka (CQ 99) - elevation of 450 m a.s.l.; broad meadows in a forest of sessile oak; Vorovo (CQ 79) - elevation of 200 m a.s.l.; spacious meadows in a forest of pedunculate oak, hornbeam and Turkey oak with linden; Čortanovci (DR 20) - elevation of 470 m a.s.l.; narrow meadows and footpaths in a forest of Turkey oak and broad-leaved yellowwood; Brankovac (DR 00) - elevation of 470 m a.s.l.; broad meadows within a sessile oak forest; Ležimir (CQ 89) - elevation of 295 m a.s.l.; Andrevlje (CR 90) - elevation of 150 m a.s.l.; Rakovački Rit (DR 00) - elevation of 80 m a.s.l.; Kamenički Park (DR 00) - elevation of 80 m a.s.l.; Astal, Direk (DR 10) - elevation of 355 m a.s.l.; a forest of sessile oak and hornbeam with European bladdernut; Tancoš, Testera (CR 90) - elevation of 150 m a.s.l.; Velika Remeta (DR 10) - elevation of 320 m a.s.l.; a forest of Turkey oak and Virgilius's oak; Kucoš, Krušedol (DQ 19) - elevation of 230 m a.s.l.; Ravne (CQ 89) - elevation of 400 m a.s.l.; Paragovo (DR 00) - elevation of 220 m a.s.l.; a forest of oak with hornbeam and beech; below Glavica (DR 10) - elevation of 300 m a.s.l.; a forest of sessile oak and hornbeam with sweet woodruff; Grgurevačka Pećina Cave surroundings, Grgurevci (CQ 99) - elevation of 410 m a.s.l.; Krčedin (DR 30) - elevation of 100 m a.s.l.; Bukovac (DR 10) - elevation of 190 m a.s.l. The specimens of jewel beetles are kept in the collection of the first author. All recorded taxa are photographed and presented in Plate 1.

RESULTS AND DISCUSSION

As a result of the research, totally 14 harmful species of jewel beetles (Coleoptera: Buprestidae) are recorded in the area of the Fruška Gora National Park (North Serbia), what is somewhat less than ¼ of all species of jewel beetles recorded in Serbia (Ćurčić et al., 2001). These 14 taxa are classified into 11 genera. All taxa are listed and illustrated (Tables 1 and 2; Plate 1), and their UTM localities in the Fruška Gora National Park are given in the Materials and Methods (Table 1, Map 1). The list of harmful jewel beetle species, the localities and dates of findings, the flight period, the number of collected specimens and the host plants are given in Table 1. The total body length, bionomy, and zoogeographical distribution

Plate 1. The images of adult jewel beetle species found in the Fruška Gora National Park.

of each of the recorded species is presented in Table 2. The most frequent species are *Ovalisia rutilans* and *Chrysobothris affinis*, with the estimated frequency of 50-100 recorded specimens per research day each (Table 1). It is necessary to pay a special attention to behaviour of these species in the future. These species are followed by *Capnodis tenebrionis*, *Coraebus rubi* (Linnaeus, 1767) and *Trachys minutus* (Linnaeus, 1758), with 10-50 specimens per research day each (Table 1). Their frequency is not concerning, with the exception in the case of *Coraebus rubi*. For this species, a more careful monitoring is needed to be performed in order to explore the possible existence of the increasing population trend. The rest of the species, nine out of 14, were found in a small number of specimens each (1-10) (Table 1). No recorded species have shown such frequency to cause obvious damage to their host plants in protected forest areas of the Fruška Gora National Park. Further researches are welcomed in order to enable expanding the list of jewel beetle species and their distribution characteristics in the protected area. Consequently, the comparisons with similar results of related, on-going researches on jewel beetles in other protected areas in the Republic of Serbia are necessary to be performed in the future.

Table 1. Jewel beetle species found in the Fruška Gora National Park, with the data on the localities and finding dates, the flight period, the abundance and the host plants.

SERIAL NO.	SPECIES	PHOTO NO.	LOCALITIES AND FINDING DATES	FLIGHT PERIOD	ABUNDANCE (NUMBER OF COLLECTED SPECIMENS PER RESEARCH DAY)	HOST PLANTS
1.	*Ptosima undecimmaculata* (Herbst, 1784)	1	Paragovo, 01.05.2007; Grgurevačka Pećina Cave surroundings, Grgurevci, 10.05.2011	MAY - JULY	1-10	*Cerasus* spp., *Crataegus monogyna* Jacq., *Prunus* spp. (Rosaceae)
2.	*Capnodis tenebrionis* (Linnaeus, 1758)	2	Krčedin, 23.06.2009; below Glavica, 17.07.2008	MAY - AUGUST	10-50	*Crataegus* spp., *Prunus* spp., *Pyrus communis* L. (Rosaceae)
3.	*Perotis lugubris* (Fabricius, 1777)	3	Krčedin, 23.06.2009; Bukovac, 28.06.2005	JULY	1-10	*Crataegus* spp., *Prunus* spp. (Rosaceae)
4.	*Dicerca alni* (Fischer, 1824)	4	Andrevlje, 25.05.2009; Brankovac, 31.05.2005	MAY - JUNE	1-10	*Fagus sylvatica* L. (Fagaceae), *Alnus* spp. (Betulaceae), *Juglans regia* L. (Juglandaceae), *Tilia cordata* Mill. (Tiliaceae)
5.	*Ovalisia rutilans* (Fabricius, 1777)	5	Andrevlje, 25.05.2009; Letenka, 08.07.2014; Vorovo, 25.06.2008; Iriški Venac, 01.06.2003	MAY - JULY	50-100	*Tilia* spp. (Tiliaceae)
6.	*Phaenops cyanea* (Fabricius, 1775)	6	Velika Remeta, 21.05.2014, 02.07.2014, 07.07.2014; Popovica, 19.05.2014	MAY - AUGUST	1-10	*Pinus* spp. (Pinaceae)
7.	*Anthaxia manca* (Linnaeus, 1767)	7	Velika Remeta, 21.05.2014; Rakovački Rit, 21.04.2008	APRIL - JUNE	1-10	*Ulmus* spp. (Ulmaceae)
8.	*Chrysobothris affinis* (Fabricius, 1794)	8	Velika Remeta, 21.05.2014; Ležimir, 11.06.2008; Tanco, Testera, 04.07.2014; IriškiVenac, 27.05.2003	MAY - AUGUST	50-100	Numerous (an extremely polyphagous species)
9.	*Coraebus florentinus* (Herbst, 1801)	9	Ravne, 28.06.2009	MAY - JULY	1-10	*Quercus* spp. (Fagaceae)
10.	*Coraebus rubi* (Linnaeus, 1767)	10	Velika Remeta, 02.07.2014; Čortanovci, 01.07.2008; Kuco, Krušedol, 07.07.2010; Krčedin, 24.06.2009; Astal, Direk, 12.06.2008	MAY - JULY	10-50	*Rosa* spp., *Rubus caesius* L., *R. fruticosus* L. (Rosaceae)
11.	*Agrilus biguttatus* (Fabricius, 1776)	11	Iriški Venac, 27.05.2003, 01.06.2003; Paragovo, 27.05.2003; Vorovo, 29.07.2008; Andrevlje, 21.06.2013; Tanco, Testera, 04.07.2014	MAY - AUGUST	1-10	*Fagus sylvatica*, *Quercus cerris* L., *Q. petraea* (Mattuschka) Liebl., *Q. pubescens* Willd. (Fagaceae)
12.	*Agrilus viridis* Linnaeus, 1758	12	Stražilovo, 13.05.2005; Ležimir, 11.06.2008	MAY - JULY	1-10	*Acer* spp. (Aceraceae), *Alnus* spp., *Betula* spp. (Betulaceae), *Salix* spp. (Salicaceae), *Tilia* spp. (Tiliaceae)
13.	*Trachys minutus* (Linnaeus, 1758)	13	Iriški Venac, 10.07.2014; Kamenički Park, 10.05.2006	MAY - AUGUST	10-50	*Acer campestre* L. (Aceraceae), *Ulmus* spp. (Ulmaceae), *Salix* spp. (Salicaceae), *Tilia* spp. (Tiliaceae)
14.	*Trachys troglodytiformis* Obenberger, 1918	14	Stražilovo, 24.04.2009	MAY	1-10	*Althaea* spp., *Malva* spp. (Malvaceae)

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Table 2. List of jewel beetle species collected in the Fruška Gora National Park, with the data on the body length, bionomy, and total distribution.

SERIAL NO.	SPECIES	BODY LENGTH AND BIONOMY	TOTAL DISTRIBUTION
1.	*Ptosima undecimmaculata* (Herbst, 1784)	7-14 mm. Preferring forest steppes, pastures and warm shrubland on escarpments.	Circum-Mediterranean
2.	*Capnodis tenebrionis* (Linnaeus, 1758)	14-28 mm. Preferring forest and rocky steppes, widely distributed in orchards and pastures used for extensive sheep grazing as well.	Circum-Mediterranean
3.	*Perotis lugubris* (Fabricius, 1777)	13-27 mm. Preferring ligneous part of roots of old, but still live fruit trees. Common in hills and mountains, but has recently become rare.	Eastern Mediterranean
4.	*Dicerca alni* (Fischer, 1824)	16-23 mm. Preferring lowland forests, swamps and wet river valleys although it can be found on sun-exposed, rather dry slopes.	Western Mediterranean
5.	*Ovalisia rutilans* (Fabricius, 1777)	11-15 mm. Frequently found under the bark of trunks or thicker branches of some lime trees, usually only on the sunny sides.	European
6.	*Phaenops cyanea* (Fabricius, 1775)	8-11 mm. Preferring both natural pine forests and pine plantations.	Euro-Siberian
7.	*Anthaxia manca* (Linnaeus, 1767)	7-11 mm. Preferring lowlands, but following big rivers.	Euro-Caspian
8.	*Chrysobothris affinis* (Fabricius, 1794)	10-15 mm. Preferring lowland and medium-altitude forests.	Western Palaearctic
9.	*Coraebus florentinus* (Herbst, 1801)	12-16 mm. It prefers lowlands and forest steppes on escarpments.	Euro-Mediterranean
10.	*Coraebus rubi* (Linnaeus, 1767)	7.5-11.0 mm. Preferring steppe and sun-exposed rocky slopes, colonizing very often ruderal biotopes.	Caspio-Mediterranean
11.	*Agrilus biguttatus* (Fabricius, 1776)	10-14 mm. Preferring light oak forests.	Western Palaearctic
12.	*Agrilus viridis* Linnaeus, 1758	4.5-10.0 mm. Extremely polyphagous species.	Euro-Siberian
13.	*Trachys minutus* (Linnaeus, 1758)	3.0-3.5 mm. Widely distibuted and rather polyphagous, without any special ecological demand.	Euro-Siberian
14.	*Trachys troglodytiformis* Obenberger, 1918	2.5-3.0 mm. Inhabits steppes, forest steppes, uncultivated dry meadows and ruderal habitats both in lowlands and on escarpments.	Circum-Mediterranean

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CONTROL OF BARK BEETLE POPULATION AT THE TARA NATIONAL PARK BY PHEROMONE TRAPS

Marko Tomic and Branko Bezarevic

PE "National Park Tara", Milenka Topalovica 3, 31 250 Bajina Basta, Serbia
branko.bezarevic@nptara.rs

ABSTRACT

Tara National Park, covering the area of 19,175 ha, represents one of the best preserved forest complexes in Serbia. It is most recognizable for Serbian spruce (*Picea omorika* Pancic Purk.), which has very narrow range of distribution and only a few natural habitats. Since the spring of 2013, forest dieback took a larger scale as consequence of extraordinary drought periods in previous years, particularly in 2012. Such dead trees and physiologically weak trees are suitable for reproduction of harmful species of bark beetles, (Coleoptera: Curculionidae: Scolytinae) on coniferous trees. Significant pests are *Ips typographus* (L.), *Pityogenes chalcographus* (L.) and *Polygraphus polygraphus* (L.) on spruce and Serbian spruce, *Pityokteines curvidens* (Germ.) on fir, *Ips sexdentatus* (Boern.) on Austrian and Scots pine, as well as *Trypodendron lineatum* (Oliv.) on spruce, fir and pine trees.

Professional teams experienced in detecting endangered trees or sites (hotspots), made an on site inventory. On that occasion there were marked suitable locations for pheromone traps. Feeding and processing the data into GIS program revealed total endangered area of 6,020 ha, where 1200 pheromone traps were mounted along with appropriate pheromones. During bark beetle flight, periodical visits and discharging of containers were done. After that was conducted counting of captured bark beetle specimens, altogether with entering and processing data. Few hundreds of bark beetles are able to kill a hundred year old tree. During our research, till end of swarming period, there were caught 33,358,102 bark beetles.

INTRODUCTION

The Tara National Park (N.P. Tara), occupying the largest part of Mt. Tara is located in the far west of Serbia, including an area bounded by the Drina River between Višegrad and Bajina Basta. The region of the N.P. Tara ranges between 43°52' and 44°02' North latitude and between 19°15' and 19°38' East longitude. The highest point of NP Tara is Kozji Rid (1591 m), and the lowest point is at 300 m. It occupies an area of 19,175 ha, and approximately 15,802 ha are covered by forests.

The climate of Mt.Tara is characterized by fresh to cool summers and quite cold winters, with small variations of annual temperature (Medarević, 2005). The climate, together with topographic and edaphic conditions favourably influenced the development of diverse flora, including forest ecosystems with autochthonous vegetation, particularly relicts. The flora of the NP Tara is characterized by remarkable diversity (Obratov, Đukić 1997).

Forests in the area of the NP Tara are among the best preserved forest complexes in Serbia. Coniferous forests predominate, especially mixed stands of silver fir (*Abies alba* Mill.), beech (*Fagus moesiaca* K. Mally/ Czeczott) and European spruce (*Picea abies* Karst.). The most common coniferous species are fir and spruce, although there are also Austrian pine (*Pinus nigra* Arnold) and Scots pine (*Pinus silvestris* L.) forests. Mt.Tara is the most recognizable for Serbian spruce (*Picea omorika* Pancic Purk.), a coniferous endemic tree with a very narrow range of distribution and only a

few natural habitats. It is an endangered species listed on the IUCN RED List (Vulnerable status).

Two outbreaks of bark beetles were recorded from 1929 to 1931 and from 1945 to 1947 in former Yugoslavia causing a loss of several millions m^3 of wood to our forestry. In 1997-2000 bark beetles endangered over 20,000 ha of forests in the Republic of Srpska, of which the majority were spruce forests. The next outbreak began in the Republic of Srpska in 2005. From 2001 to 2005, about 700 ha of pure spruce forests were devastated in Serbia on Mt.Stara Planina, as a result of outbreaks of *Ips typographus* and *Pityogenes chalcographus* (Mihajlović, 2008). Since the spring of 2013, forest dieback took a larger scale, both in individual trees and large groups of trees of all ages and in all coniferous species: fir, spruce, pine and Serbian spruce. The most important factors that influence its occurrence are extremely dry months and high summer temperatures in recent years, particularly in 2012 (Mihajlović, 2013).

Such dead and physiologically weak trees represent suitable material for the reproduction of harmful species of bark beetle (Coleoptera: Curculionidae: Scolytinae) on coniferous trees. When their population is highly

Figure 1. Dieback of spruce

Figure 2. Dieback of fir

increased, and such material starts to run out, they try to inhabit completely healthy trees as well.

This research is focused on the analyses and control of *Ips typographus* (L.), *Pityogenes chalcographus* (L.) and *Polygraphus polygraphus* (L.) in spruce and Serbian spruce, *Pityokteines curvidens* (Germ.) in fir, *Ips sexdentatus* (Boern.) in Austrian and Scots pine, as well as *Trypodendron lineatum* (Oliv.) in spruce, fir and pine trees. The aim was to prevent an outbreak of bark beetle populations in 2014, and to control the bark beetles in subsequent years by pheromone traps.

MATERIAL AND METHODS

The study was conducted on an area of 6,020 hectares, at altitudes ranging from 900 to 1500 m. To a largest extent it included a selection of the stands of beech (32.4%), fir (39.1%) and spruce (15.6%), followed by the localities with Serbian spruce and some stands of black and white pine. First, reconnaissance of the terrain was performed. The infested trees or groups of trees were recorded (hot spots) in the field and at the same time suitable places were also selected to set

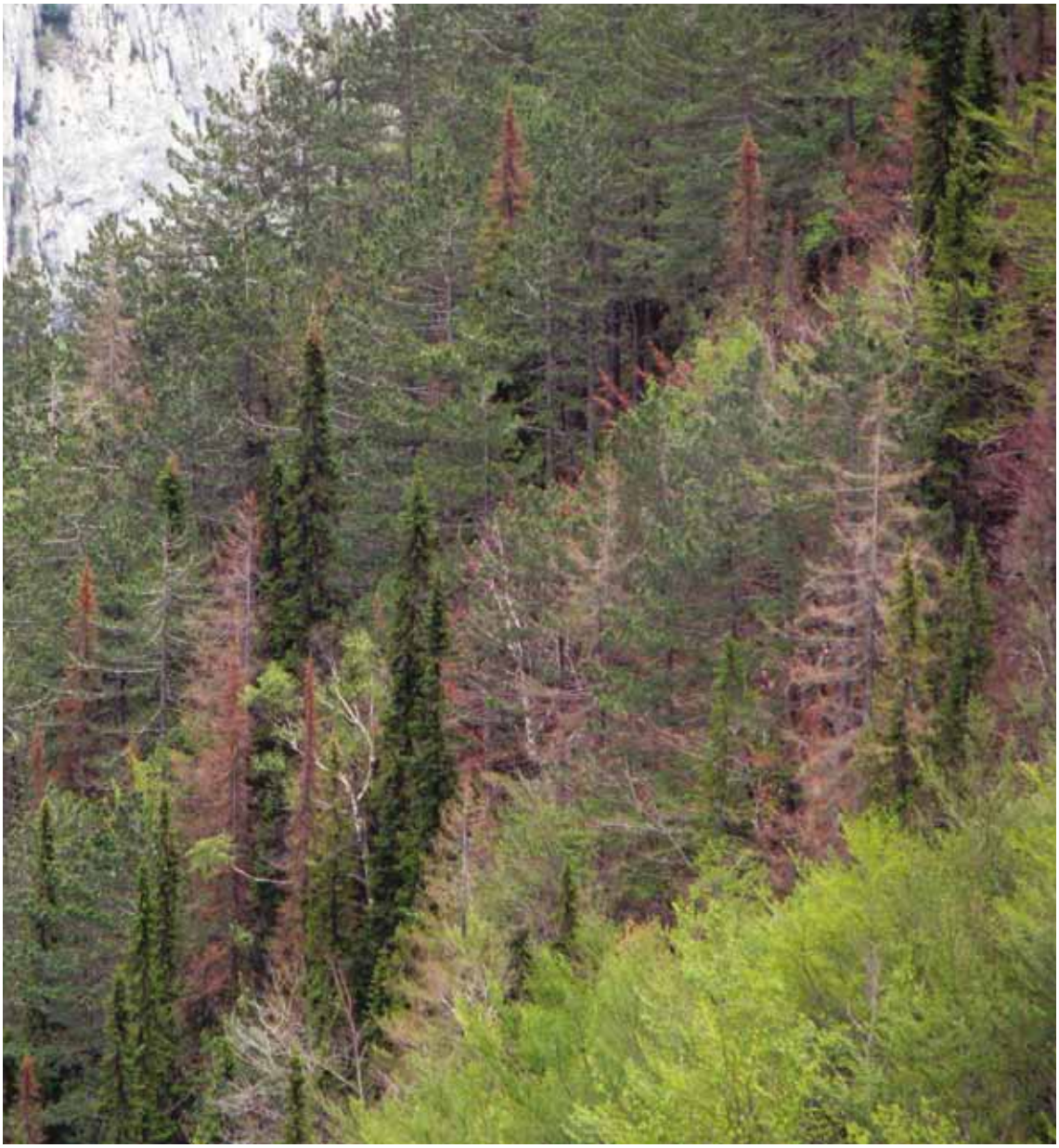


Figure 3. Dieback of Serbian spruce

pheromone traps. After that, these locations were marked using a GPS device. Data from all GPS devices were entered into ArcGIS 9.3, which was used to add a small number of points established via ortho-photo images, as a complement to the anticipated number of traps. In this manner, a network of points, which represent the future location of traps (Figure 4) was obtained. In the ArcGIS program, each point was associated with a unique serial number and type of pheromone that will be used at this location, so that all points could then be returned to the GPS devices.

For the purposes of research and monitoring cross-barrier traps were used with dry containers, type THEYSOHN®, which were placed on the holders of a crossbar with a 3x5 cm cross section. Before the expected incidence of adults, a total of 5 pheromone traps were set in the lowest elevation of endangered forests. According to Mihajlović (2008), when the test traps catch the first adult, setting of other traps on the ground should follow. In the period from 18th to 28th March 2014, a total of 1200 traps were set, together with the respective pheromones in previously marked locations using GPS devices. The traps were arranged in such a way that in less vulnerable parts, one trap covered about 15ha, and in more vulnerable parts the area of from 0.5 to 5 ha.

The traps were controlled and emptied every 10-15 days. The trapped beetles that were collected in the field were counted by the volumetric method in a calibrated container. In one ml (1 cm^3) there were approximately 40 individuals of *Ips typographus*, i.e. 400 individuals of *Pityogenes chalcographus* (Svestka, 2014). In the case of traps in which dispensers for both of these species were placed together, the separation was performed using a sieve with a 1,5x2 mm mesh size. For *Pityokteines sp.* it was empirically found that in one ml there were approximately 200 individuals. The counting data were further processed in Microsoft Excel 2007. After that, the values obtained were entered in ArcGIS. This resulted in a map of the vulnerable area, according to the severity of the attack, which can serve as the basis for forecasting and monitoring in the coming years.

Pheromone dispensers were replaced in mid-June, and the process of monitoring lasted until the end of September.

RESULTS

During our research, till end of the swarming period, a total of 33,358,102 bark beetles were caught. During the first swarming a total of 10,072,602 bark beetles were trapped, whereas during the second swarming that number was 23,285,500.

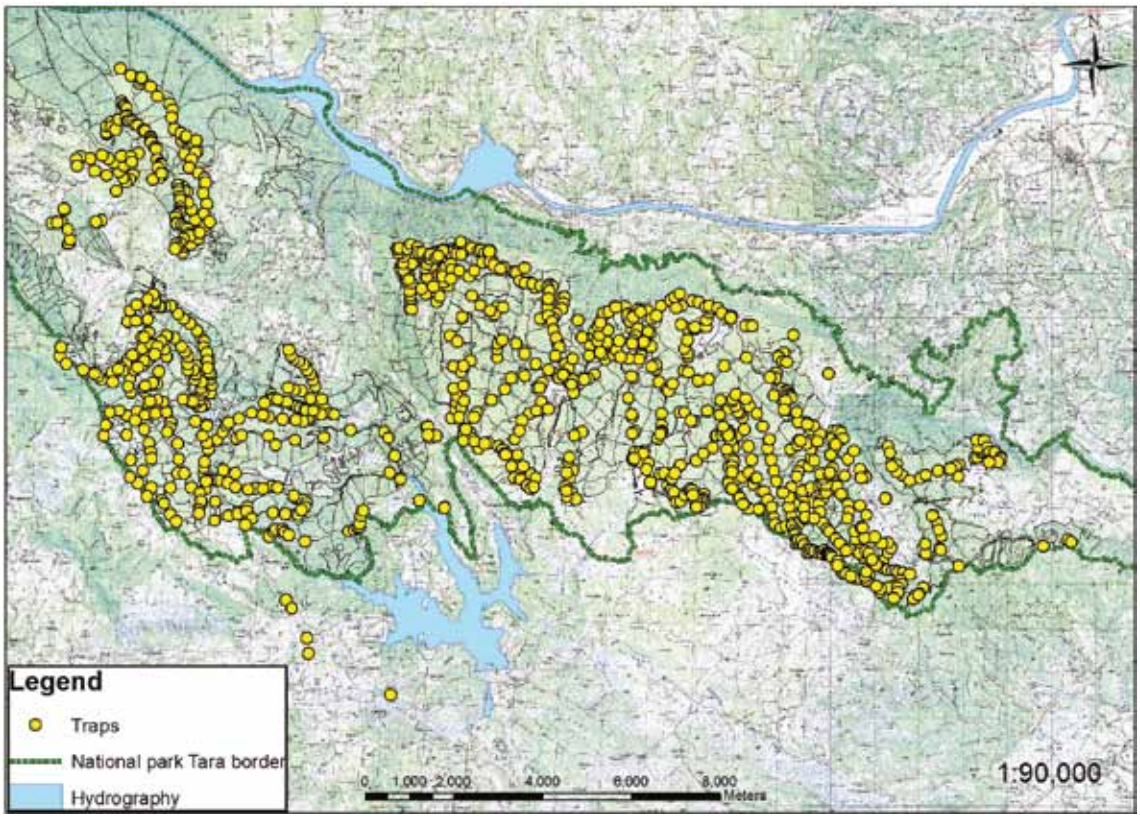


Figure 4. Distribution of pheromone traps in the field

It is necessary to point out that certain traps recorded trapping of up to 60,000 individuals of *Ips typographus*, i.e. about 300,000 individuals of *P. chalcographus*.

In Figure 5, the red dots represent traps that caught over 4000 individuals of *Ips typographus* in the springtime swarming, representing a severe attack (Karadžić et al., 2011). After the springtime swarming, traps from the sites where no significant catches were recorded were relocated to the places where the traps recorded massive catches.

Table 1. Number of pheromones by species in the springtime swarming and summertime swarming. PC-ECOLURE was set together with IT-ECOLURE in 253 traps before the springtime swarming and in 228traps before the summertime swarming.

Type of pheromone	Bark beetle	Number of dispensers in the springtime swarming	Number of dispensers in the summertime swarming	In total
IT-ECOLURE	*Ips typographus*	588	612	1200
PC-ECOLURE	*Pityogenes chalcographus*	253	347	600
CURVIWIT®	*Pityokteines curvidens*	482	395	877
POLYWIT®	*Polygraphus polygraphus*	120	62	182
SEXOWIT®	*Ips sexdentatus*	8	9	17
TRYPOWIT®	*Trypodendron lineatum*	2	3	5

Table 2. The number of trapped bark beetles in the springtime and summertime swarmings by species

Bark beetle	Number of trapped bark beetles in the springtime swarming	Number of trapped bark beetles in the summertime swarming	The total of trapped beetles
Ips typographus	4216737	4535226	8751963
Pityogenes chalcographus	5829808	18750216	24580024
Pityokteines curvidens	15107	243	15350
Ips sexdentatus	4138	68	4206
T. lineatum	6559	0	6559

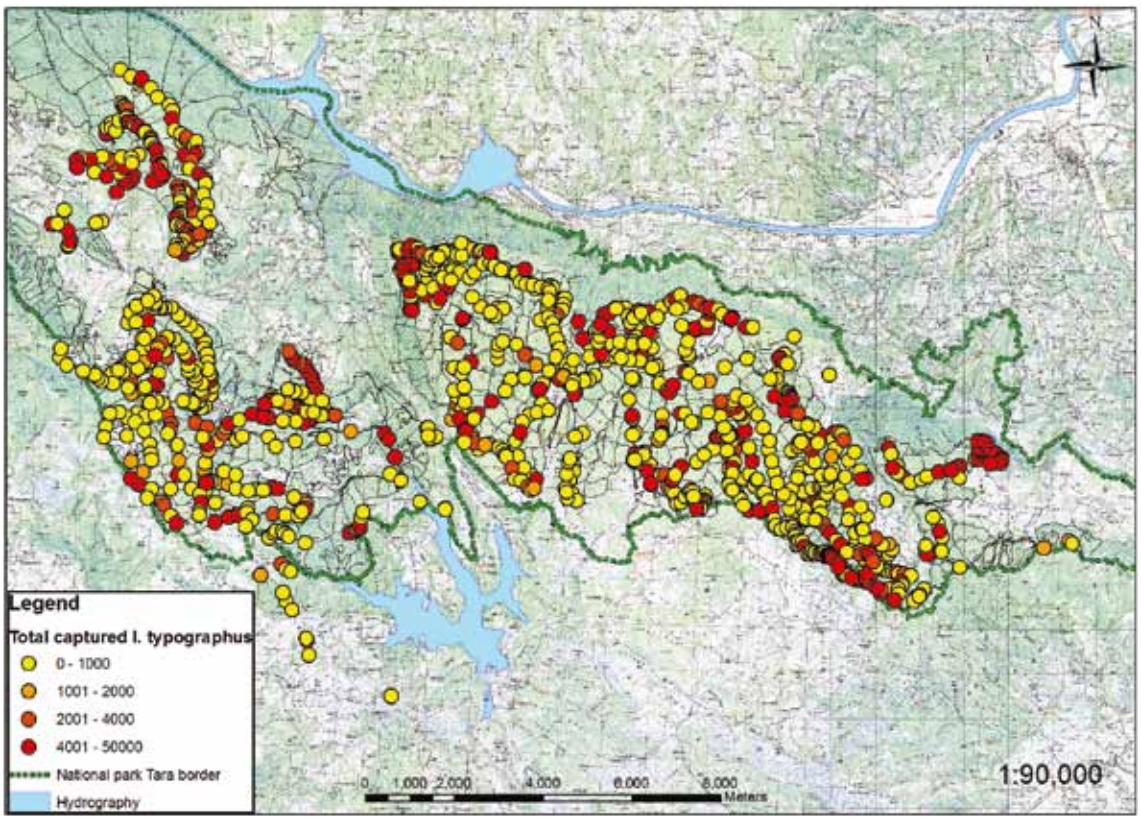
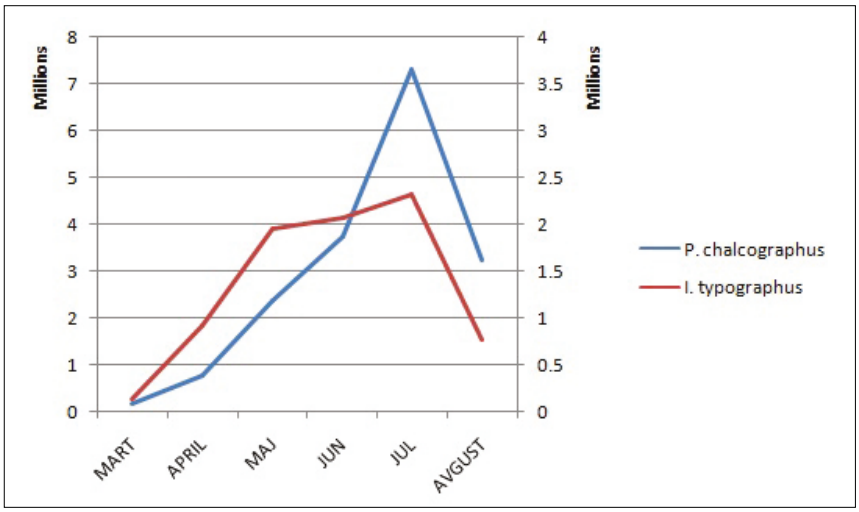


Figure 5.



Graph 1.

It can be noticed in Graph 1 that the maximum catches of *I. typographus* and *P. chalcographus* occurred in mid-July. It is important to note that, the catches from traps that were previously relocated or supplemented with other types of pheromones were not calculated for the months of June, July and August.

DISCUSSION

According to the results, it can be said that the biggest catch of *I. typographus* and *P. chalcographus* were recorded exactly in the places of severe dieback of spruce trees at the beginning of 2013. These are habitats with shallow and skeletal soil, west-south exposure, especially in ridge locations on limestone substrates.

Faccoli and Stergulc (2004) determined a strong correlation between the number of captured insects per trap and the number of trees attacked by *I. typographus*, fixing a damage threshold of about 8,000 insects per trap per year, which is very similar to the value of 10 000 found in forests of Swedish spruce. Dr. Brutovsky prepared a special scale for the evaluation of the damage threshold by both *I. typographus* and *P. chalcographus* in Slovak conditions (Zubrik *et al.* 2006). For *I. typographus* 5,500 – 8,000 beetles per trap per year is considered the limit and for *P. chalcographus* it is 40 – 60,000 bark beetles per trap per year. We can only conclude that the conditions of this study by far exceeded these threshold values.

The catches of *Pityokteines sp.* and *Polygraphus polygraphus* were very low, so a possible malfunction of the dispenser can also be assumed, although that requires further investigation. Only a few hot spots where *Ips sexdentatus* was present were found, so consequently a small number of traps with dispensers for this species were set. Traps designed to capture *T. lineatum* were placed at a landing used for log keeping.

During 2013, 27,823 m^3 of fir and spruce wood were removed with rehabilitation measures and in 2014, the measures included 12,741 m^3 of fir and spruce, which represents a decrease of about 54%. It is obvious that there was a significant reduction in dieback compared to the year before. However, the weather conditions during that year, which are likely to have affected the recovery of coniferous forests, should also be mentioned.

Pheromone traps should be part of integrated protection together with the debarking of logs and timely removal of the suitable material from the forest. Together with GIS technology, which greatly facilitates their installation and control, they should serve as a means of continuous monitoring of bark beetles in coniferous forests of Serbia, especially because of the increasingly frequent extreme climatic conditions occurring in recent years.

On the basis of the experience from previous research, a need arose for the creation of a „health card" of the National Park Tara in the form of a digital map representing not only a map of vulnerability to the outbreaks of bark beetles, but also to other pests and diseases that occur as a result of climate change.

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COMPARATIVE TRIALS OF FOUR POTASSIUM PHOSPHITE FORMULATIONS AGAINST CHESTNUT INK DISEASE BY TRUNK INJECTION

Elisa Dal Maso[1] and Lucio Montecchio[1*]

[1]Università degli Studi di Padova, Dipartimento Territorio e Sistemi Agro-Forestali, V.le dell'Università 16, I-35020 Legnaro, Italy
[*]Corresponding author: L. Montecchio. E-mail address: montecchio@unipd.it

ABSTRACT

Ink disease, caused by *Phytophthora cinnamomi* and *P. cambivora*, is one of the most destructive diseases affecting *Castanea sativa*. Currently, disease control requires careful integrated chemical and agronomic measures. Trunk injection with potassium phosphite was shown as curative in reducing symptoms expression but little is known about the ideal formulation. In this research, fifty asymptomatic sweet chestnuts were inoculated with a local strain of *P. cinnamomi* isolated from an infected chestnut. Subsequently, trees were injected with four formulations of potassium phosphite. In comparison with water treatment, after 50 days the growth of the necroses was significantly slowed down only by one formulation, consisting in potassium phosphite added with a micronutrient solution. The results increase the knowledge base on the efficacy of endotherapic treatments against chestnut ink disease.

Keywords: Chestnut ink disease; *Phytophthora*; trunk injection; potassium phosphite; micronutrients.

INTRODUCTION

Phytophthora cambivora (Petri) Buism. and *P. cinnamomi* Rands are soil-borne pathogens responsible of the so-called chestnut ink disease (CID), one the most destructive diseases of sweet chestnut (*Castanea sativa* Mill; Vettraino et al., 2005; Vannini et al., 2010). CID can be prevented or controlled by integrated chemical and agronomic measures and protocols (IPM, IPC; Bounous and Abreu, 1998; Brasier, 1999). Phosphonates (potassium phosphite, PP) are effective *in vitro* and *in planta* against both pathogens (Coelho et al., 2005; Hardy et al., 2001; Wilkinson et al., 2001). In comparison with PP foliar treatments (Gouveia et al., 2010), trunk injection (Gentile et al., 2009) can lead to less or none phytotoxic effect (Garbelotto et al., 2007), but little is known about ideal concentration and formulation.

The aim of this study was to ascertain best performing PP formulates to control CID in artificially infected trees by trunk injections.

MATERIALS AND METHODS

Isolation of *Phytophthora cinnamomi*

In a CID infected chestnut forest (Northeastern Italy; 45° 47' N; 11° 50' E; Scattolin et al., 2012), 10 subcortical samples (~ 15 cm^3) were collected from both roots and trunk base of a symptomatic tree. Samples were immediately processed by means of lateral flow tests for *Phytophthora* spp. (Forsite Diagnostics Ltd., Surrey, UK). Furthermore, four equidistant soils cores (10 x 20 cm, 45 cm in depth, 50 cm from the trunk) were processed by baiting (Jung et al., 1996;

Franceschini, 2011) using fresh *C. sativa* leaves as baits. *Phytophthora*-like colonies (Erwin and Ribeiro, 1996) not producing sporangia were treated to stimulate fructifications according to Halsall and Forrester (1977).

Among the seven morphologically comparable pure cultured isolates obtained, one was definitively selected as representative, and processed with PCR (Kong et al., 2005), together with an official *P. cinnamomi* strain (CBS 144.22). Cox1 primers (FM84 and FM83 for amplification; FM50 for sequencing) were used for polymerase chain reaction (Martin et al., 2014). A sample of 10 µl of the PCR product was electrophoresed on a 1% agarose gel together with MassRuler DNA Ladder Mix (Thermo Scientific, US), stained with Green Gel Plus™ (Fisher Molecular Biology, Società Italiana Chimici, Roma) and imaged using UVIpro Gold Gel Documentation System (UVItec, Cambridge, UK). The amplified products, purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA), were sequenced by BMR Genomics (Padova, Italy). BLASTn research was then performed on the obtained sequences (http://www.ncbi.nih.gov/BLAST, accession date 02/06/2014).

Artificial inoculation and trunk injection

The trial was performed in a chestnut coppice (45° 54' 41'' N; 12° 2' 10'' E), where fifty asymptomatic stems ranging from 7.5 to 14.5 cm (ave. 9.9 cm) dbh belonging to neighboring stumps were selected. In June 2014, every stem was inoculated 150 cm above the collar according to Dal Maso et al. (2014) with one 7 mm diam. plug of the strain previously isolated, grown on PDA for 7 days at 24±1 °C in the dark. After 20 days, the edges of carefully debarked necroses were photographed and then accurately marked in GIMP v. 2.8. A script in MATLAB v. 8. was created for unwrapping of cylindrical trunk photos, then areas were measured in ImageJ v. 1.46r (Wajne Rasband, National Institutes of Health, USA; Dal Maso et al., 2014).

According to both tree diameter and necrotic area, stems were organized into five comparable groups (Peterson et al., 2009), to be injected with different commercial products diluted in water: a) FOSFISAN 35% v/v (122.5 g/L H_3PO_3 final concentration), b) FOSFISAN 70% v/v (245 g/L H_3PO_3; Franceschini, 2011), c) FOSFISAN 35% v/v plus 20% v/v CONQUER (1 g/L allicin), d) FOSFISAN 35% v/v plus 0.1% v/v AGROVIT L micronutrients solution (Table 1), e) water as control. Stems were injected 190-200 cm from the base, in 2-3 equidistant points never above the inoculation site, for a total amount of liquid corresponding to 1 mL/cm dbh. To avoid the production of drill holes, a hollow bladed, manual injection tool was used (BITE, University of Padova pat. n. WO2013010909-A1; Montecchio, 2013; Fig. 1).

Figure 1. Trunk injection treatment

Fifty days from treatment, the necrotic areas were re-measured and compared with the ones assessed on the day of treatment, computing relative ratios (Dal Maso et al., 2014).

Furthermore, to verify the presence and vitality of the fungus, two equidistant 3 mm^3 wood samples were collected along the edge of each inoculation point, plated on PDA and incubated for 7 days at 24 ± 1°C in the dark. Isolations were scored as positive when fungal cultures exhibited the typical *P. cinnamomi* morphology (Erwin and Ribeiro, 1996).

Table 1. Commercial products used

Commercial product	Active ingredient and strength	Manufacturer
FOSFISAN	P_2O_5 30 % + K_2O 20 %	Agrofill by Adriatica S.p.a.
CONQUER	Allicin 0.5 %	JCA Limited
AGROVIT L	Soluble B 0.2 %; Soluble Cu 0.5 %; Soluble Fe 0.4 %; Fe EDTA 0.4 %; Soluble Mn 1 %; Soluble Mo 0.02 %; Soluble Zn 1 %	Agrofill by Adriatica S.p.a.

Statistical analyses

Normality and homogeneity of variance were checked with Shapiro-Wilk Normality Test and Levene Test ($p>0.01$ and $p>0.05$), respectively, then Anova ($p<0.05$) and Multiple Comparison (Tukey HSD, $p<0.05$) were used to evaluate possible differences in R cran.

RESULTS

Isolation of *Phytophthora cinnamomi*

The lateral flow test confirmed the presence of *Phytophthora* spp. Baiting essay allowed to obtain a strain morphologically identified as *P. cinnamomi,* confirmed by molecular analysis (best match sequence, *Phytophthora cinnamomi*; bit-score, 1130; E value, 0; similarity, 100 %; accession number, KC609419.1; Fig. 2).

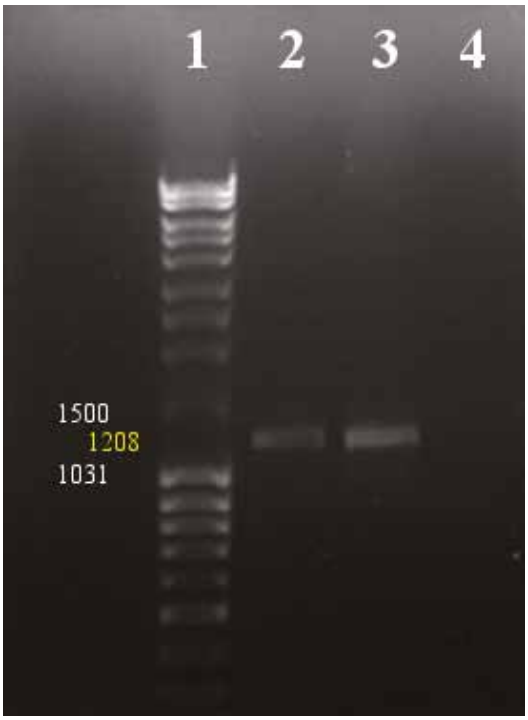

Figure 2. Agarose gel of PCR samples after electrophoresis. PCR products are as follows: lane 1, marker; lane 2, *P. cinnamomi* isolated with baiting; lane 3, *P. cinnamomi* CBS 144.22; lane 4, negative control.

Artificial inoculation, trunk injection and statistical analyses

All the inoculation points showed the development of necrotic areas. Fifty days after treatment, none of the products stopped the necrosis growth and the pathogen was successfully reisolated from their edges.

When compared with water injection, PP 35% reduced the growth of the necrosis by 65.5%, PP 70% by 62.07%, PP 35% plus allicin by 49.2% and PP 35% plus micronutrient by 84.98% in average (Anova, $p<0.01$; Shapiro-Wilk Normality Test $p=0.038$; Levene test $p=0.173$; Fig. 3). Multiple Comparison analysis indicated that the necroses' growth was significantly slowed down only by PP 35% plus micronutrients in comparison to the control ($p<0.05$; Fig. 3) and that there were no significant differences with control for all remaining treatments ($p>0.05$; Fig. 3).

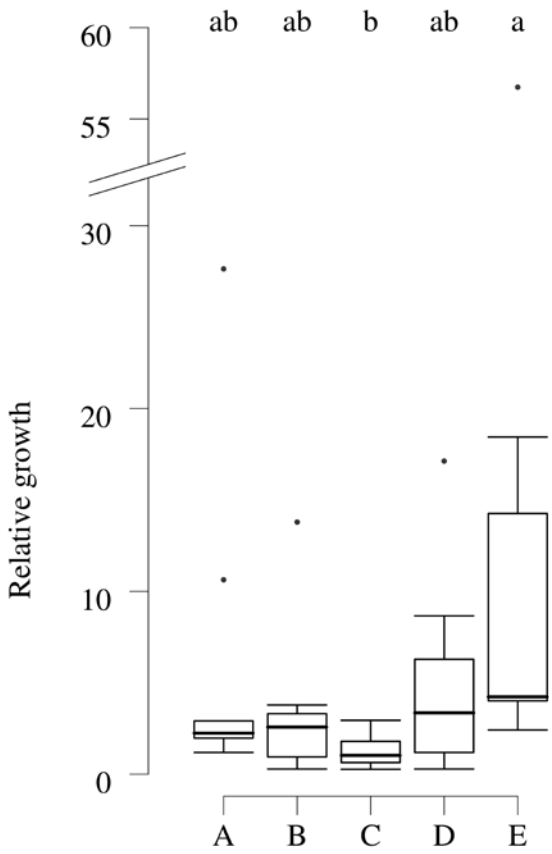

Figure 3. Differences in the relative increase of the necrotic areas 50 days from the treatment. A = Potassium phosphite 35 %; B = Potassium phosphite 70 %; C = Potassium phosphite 35 % plus micronutrient solution 0.1 %; D = Potassium phosphite 35 % plus allicin solution 20%; E = Control.

DISCUSSION

Best results were achieved by PP 35% added with the micronutrient solution. This could be due to a direct effect of single components, being the efficacy against *P. cinnamomi* of molybdenum, ferric and copper ions recognized from a long time (Halsall, 1977; Halsall and

Forrester, 1977; Keast et al., 1985; Coelho et al., 2005). Moreover, micronutrients and PP could act synergistically (Darvas et al., 1984; Bezuidenhout et al., 1987), or micronutrients could have positively influenced the plant defense response, as systemic protection could be attributed to the nutrients increasing plant cell resistance (Reuveni et al., 1997; Simoglou and Dordas, 2006; Frenkel et al., 2010).

Neither the two PP concentrations nor the one added with allicin significantly reduced the growth of *P. cinnamomi*. This is partly in contrast with the results obtained by Tamietti and Valentino (2005), but it could be mainly explained by the PP concentrations tested or by the higher pathogenicity of the fungal strain used. The unexpected effect of allicin addition, in contrast with other researches on *Phytophthora* spp. (Ke-Qiang and van Bruggen, 2001; Portz et al., 2008; Hearst et al., 2013), could be due to its main upwards translocation (Dal Maso et al., 2014).

The results of this study implement the knowledge base on endotherapic treatments against CID, indicating that the addition of micronutrients to PP directly delivered into trees can significantly slow down the development of the disease. Further planned investigations will assess the efficacy of new formulations, also in a preventive approach, particularly useful in outbreaks containment.

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REPRODUCTIVE POTENTIAL OF THE POPLAR LEAF BEETLE (*Chrysomela populi* L. 1758) UNDER DIFFERENT TEMPERATURES

Melinda Váradi and Katalin Tuba

University of West-Hungary
Institute of Silviculture and Forest Protection
H-9401 Sopron Pf. 132
varadi.melinda@emk.nyme.hu

ABSTRACT

Poplars and willows are the most frequent tree species as energy crops in Hungary. In other European countries the activities are also focused on these fast growing species. The poplar leaf beetle (*Chrysomela populi* L. 1758) is one of the most abundant and important defoliator pests in these short rotation forests. It occurs usually in young plantations and nurseries, causing damages. It is widespread all over Europe. In these cases the most important duty of the forestry management is to ensure and also to optimize the mean annual growth. An intensive plantations usually are monoclonal and due to that they are vulnerable on attach by insects therefore successful forest management cannot carry out in these fields without plant protection.

In this work the fecundity of the poplar leaf beetle was investigated under laboratory conditions. The hibernated adults were collected in March 2014 before the growing season. During the examination the developmental characteristic of 30-30 individuals were observed in three different temperatures (20°C, 25°C, 30°C) and under 16:8 photoperiod. The adults were raised on each temperature, feeding on the same food (leaves of Populus x euramericana cl. Pannonia). We measured the weight changes of the adults in each third day, as well as before and after the oviposition, the number of eggs by each female.

The results represent the egg number of each egg mass, the average time among the ovipositions. They showed that the temperature has significant influence on the fecundity of the poplar leaf beetle.

Keywords: *Chrysomela populi*, herbivore, overwintering generation, reproduction ability, oviposition, egg mass

INTRODUCTION

The poplar leaf beetle (*Chrysomela populi* L. 1758) is one of the most important defoliator pest in the short rotation forests all over Europe. *Ch. populi* causes serious problems in the nurseries as well as in the young forestations and plantations, too (Augustin et al, 1993). Many of the chrysomelids are responsible widespread for serious agricultural and forest damages. These pests are every time phytophagous species, the pupas and the imagoes both are feeding on the leaves of their host plant (Lopatin & Nesterova, 2005). In Middle Europe about 50 *Chrysomela* species are causing losses by their feeding on the shoots of trees belonging to the family of *Salicaceae* (Urban, 1997).

The use of fast growing species for bioenergy has attracted the attention, mainly in the European countries in the last decades. In these cases the most important duty of the forestry management is to ensure and also to optimize the average annual growth. The poplars (*Populus* spp.) and the willows (*Salix* spp.) are the most common trees beside black locust (*Robinia pseudoacacia*) in short rotation coppice (SRC) in Hungary (Rédei et al, 2010).

An intensive and ordinarily monocultural planting is vulnerable when are attacked by an insect, therefore successful management cannot be carried out in these fields without plant protection (Björkman et al, 2004). That is why it is so important to get to know more about this pest's copulation and fecundity.

The main purposes of this study was to obtain more knowledge:

1. the number of copulations of the poplar leaf beetle (*Chrysomela populi*),

2. amounts of eggs they produce under their lifetime,

3. the weight alternation by the male and female imagoes,

4. under three constant temperatures (20°C, 25°C, 30°C).

MATERIAL AND METHODS

Beetles (imagoes from the overwintering generation) used in this study were collected on one occasion (28th March 2014) after their emergence from winter hibernation. The samples were also collected from a population near Sárvár in Hungary (Nursery of Bajti). These beetles were shipped to the Institute of Silviculture and Forest Protection, University of West- Hungary in Sopron, where they were separated in sexes and put into two glass bottles. The matured adult beetles were kept at an average temperature of 20°C and fed with fresh poplar shots until they were used in the temperature experiment. Random pairs were made from the samples and the imagoes were placed in transparent plastic containers measured 15 x 7 x 3 cm. (Figure 1 and 2)

During the trial the developmental characteristic of 30-30 individuals were observed at three different temperatures (20°C, 25°C, 30°C) and under 16:8 photoperiod. The adults were feeding at each temperature on the same food (leaves of *Populus* x *euramericana* cl. Pannonia). Through the daily monitoring, the date of the copulation, the number of eggs and the weight changes were recorded by 30 pairs. In total, 60 beetles were examined, 10 pairs per each temperature level. The imagoes were introduced to climate chambers (CO2 Growth Chamber, model JSPC-300C2) set at constant temperatures of 25°C and 30°C. The third temperature was ensured in an insect hatchery held at 20°C. Weight changes of the imagoes were recorded with an analytical lab balance.

All data were subjected to statistical analysis with the help of Statistica 12. To describe the basic feature of data in the study we used descriptive statistic and we created Box-and-Whiskers Plots.

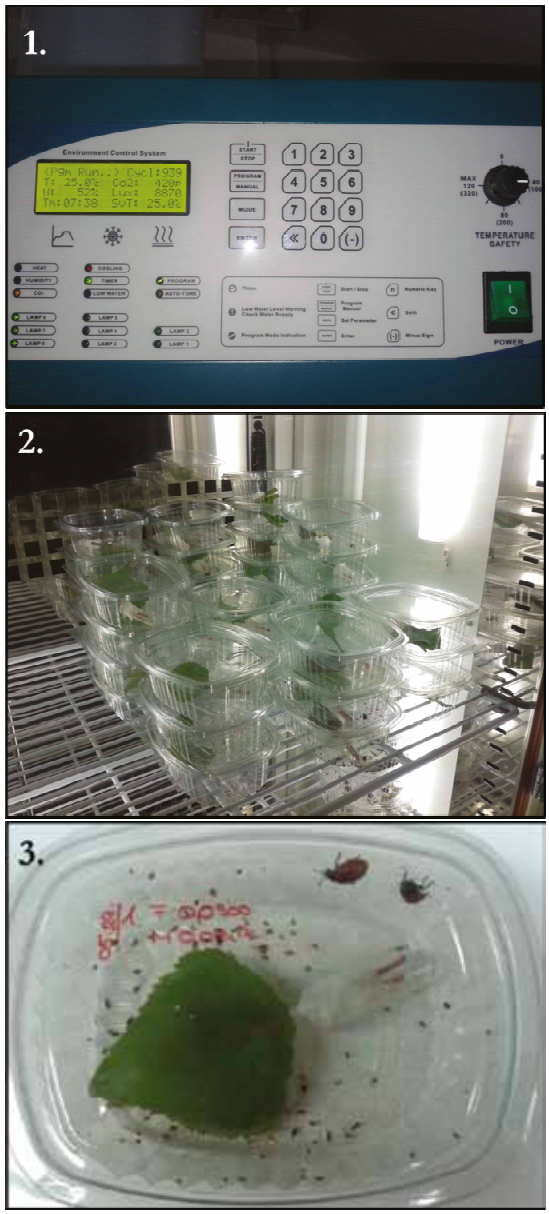

Fig. 1-3. The incubator and the samples reare in plastic boxes

RESULTS

According to our results there are differences in the egg production of *Ch. populi* among when tested on three temperatures (4. Fig).

One female from the wintering generation is able to produce eggs 1-6 times under her lifespan. The number of ovipositions were in inverse ratio to the increasing temperature, so imagoes on higher temperature are able to produce less times.

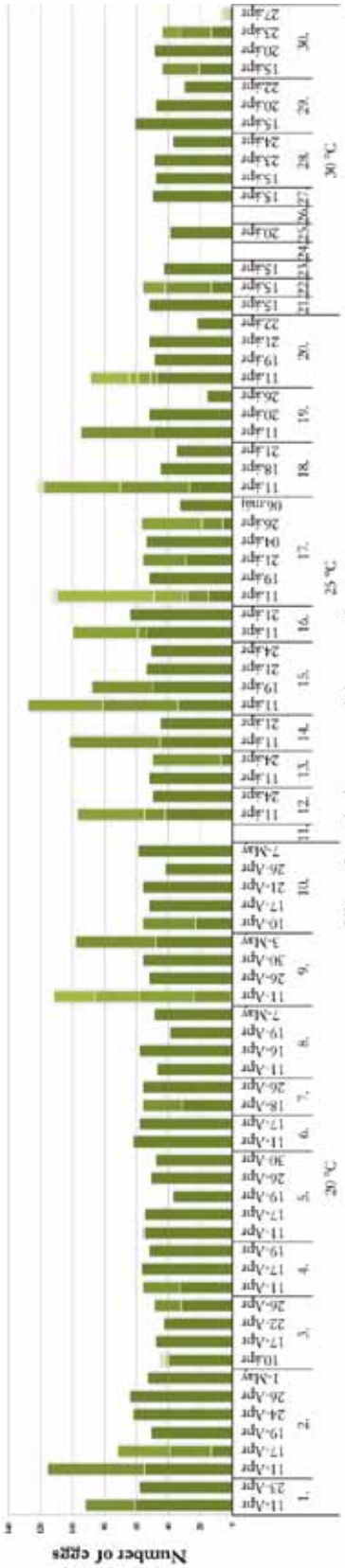

Fig. 4. The reproductive potential of the overwintering generation under 20-25-30 °C
The columns symbolises the egg laying.
The tones of the green show the numbers of egg masses.

By the size of these egg masses we did not find singificant differences among the temperatures, but when we examined the egg's amount by one oviposition the three groups separated heavily from each other (5., 6. & 7. Fig). Imagoes raised on 25 °C produced double as many eggs as pairs on 30°C. The most significant differences were observed between the lowest and the highest temperatures. In 20°C and 25°C the number of eggs were three times bigger than in the extreme high third temperature (30°C).

Variable	Descriptive Statistics (Spreadsheet1)				
	Valid N	Mean	Minimum	Maximum	Std.Dev.
20 °C	55	38,8	1,0	64,0	19,9
25 °C	60	30,4	1,0	64,0	20,5
30 °C	25	26,2	1,0	61,0	20,0

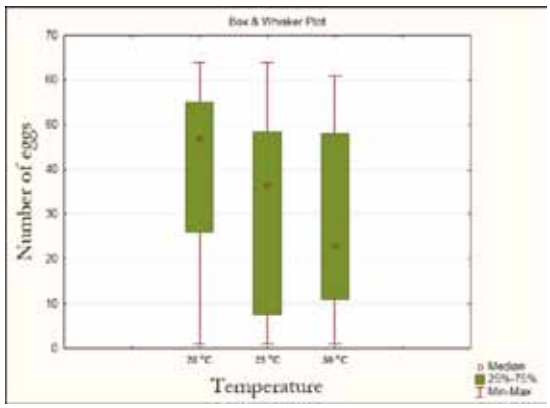

Fig. 5. Box and Whisker Plot for the egg masses

Variable	Descriptive Statistics (Spreadsheet1)				
	Valid N	Mean	Minimum	Maximum	Std.Dev.
20 °C	37	58,8	37,0	116,0	17,8
25 °C	28	65,2	16,0	128,0	30,0
30 °C	15	43,7	6,0	61,0	12,9

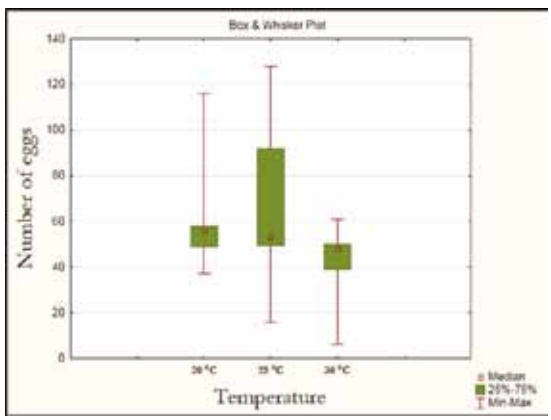

Fig. 6. Box and Whisker Plot of the together borned eggs

Variable	Descriptive Statistics (Spreadsheet1)					
	Valid N	Mean	Sum	Minimum	Maximum	Std.Dev.
20 °C	10	217,4	2174,0	112,0	418,0	96,0
25 °C	10	182,5	1825,0	0,0	366,0	103,9
30 °C	10	65,6	656,0	0,0	143,0	54,2

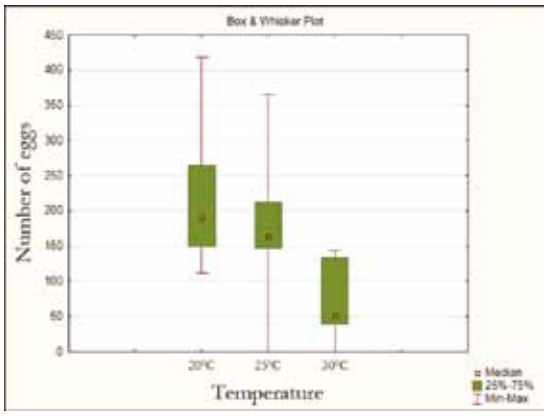

Fig. 7. Box and Whisker Plot for the total egg producion on the same temperature

The weight alternation by the sexes was measured on each temperature, but unfortunately on 30°C we could not get valuable data because of the imagoes short life. On the 8. and 9. Figures we represent the weight alternation by two randomly chose pairs on 20° and 25°C.

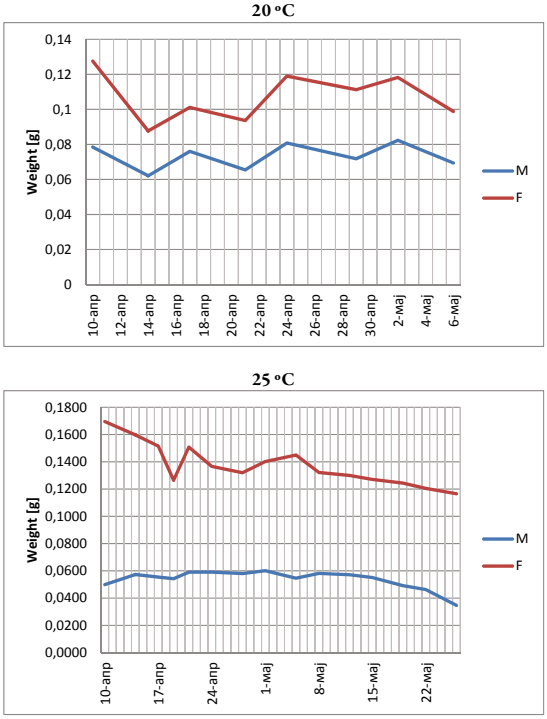

Fig 8. & 9. Weight alternation by two randomly choosed pairs on 20°C and 25°C

The sexual dimorphism appears very spectacularly in the average weight of the imagoes. While the values of the males alternate around 0,07 g the females are usually bigger (in average 0,10 g). Less difference was observed between the weight of the two sex under 25 °C than on the lower temperature, which may sing that the higher temperature affect the across the feeding the development of the imagoes.

DISCUSSION

Heat stress can induce both physiological and behavioral changes by the insects (Krebs & Loeschecke, 1994), but may result in morphological changes also (Andersen et al, 2005). That is why it is important to know, that a certain pest how behaves under different conditions?

Regarding our research, the temperature influences the fecundity and development of the poplar leaf beetle as well as its damages. The number of ovipositions is less on higher temperature. We observed much shorter lifespan in 30 °C but under this extreme condition not only the temperature plays a significant role in the imago's feeding.

It is known, that eggs are laid on the abaxial face of leaves in groups (clutches). The female prefer deposit the eggs in one group, but under laboratory conditions she divide the clutch into 2 or more groups, also in these cases the average number of eggs in one clutch is lower than in nature (Urban et al, 2006). Urban et al. observed, that number of eggs in one group change 1 to 68. According to Tillesse et al. (2007) one clutch consists of 15-65 egg.

In our research the eggs numbers varied between 1-64 by one clutch. On several occasions we observed record extreme high egg numbers, but in these cases the females laid not in a single egg mass, she composed 1-9 separated groups.

Correlate with the climate change many of the species are reacting for the changing environmental conditions. The future climate, especially rising temperature may operate several features of these pests. We need to carry out further investigations to examine the effect of other abiotic factors (drought and precipitation) on the feeding and reproduction of this pest.

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Lymantria dispar MULTICAPSID NUCLEAR POLYHEDROSIS VIRUS AND *Entomophaga maimaiga* - SIGNIFICANT BIOLOGICAL AGENTS OF THE GYPSY MOTH CONTROL IN THE FORESTS OF CENTRAL SERBIA IN THE PERIOD 2010 – 2014

Mara Tabaković-Tošić
Institute of Forestry, Belgrade, Serbia
E-mail: mara.tabakovic@gmail.com

ABSTRACT

During the period 2010-2014, a high mortality rate of old larval instars of gypsy moth (*Lymantria dispar*) was reported in beech forest complexes in some regions of Central Serbia in the culmination phase of new pest outbreak. During the field research clear symptoms of disease, caused by *Lymantria dispar* multicapsid nuclear polyhedrosis virus (*Ld*MNPV) were detected during field studies. Laboratory microscopic studies of the dead gypsy moth larvae of different larval instars revealed the presence of NPV occlusion bodies and conidiospores, and azygospores of the entomopathogenic fungus *Entomophaga maimaiga*. The mortality caused by *Ld*MNPV reached up to 20% and this caused by *E. maimaiga* – up to 100%.

Key words: *Lymantria dispar*, Serbia, classical biological control

INTRODUCTION

Biological control is the utilization by man of natural enemies for the (regulative) reduction of pest populations. Biological control, like silvicultural control, is applied ecology. A forest is a relatively undisturbed and lasting environment in which a complex network of regulating biotic factors can develop. Classical biological control is simply a special case of a general pattern in which populations are regulated by density-dependent processes, a major class of which involves predator-prey or parasitoid-host interactions. Unlike other biological cntrol strategies, conservation biological control does not require the introduction or augmentation of natural enemies. Instead, it relies on modification of the environment or management practices to protect and encourage natural enemies that are already present within the system. This improves their ability to control pest populations in a reliable way and is only possible if the biology, behaviour and ecology of both the pest and their natural enemies are understood (Hajek, 2004; Pell et all., 2010; Pimentel, 2008).

Naturally occuring enthomopathogens are important regulatory factors in insect population. Entomopathogenic organisms, various types of viruses, microsporidia, bacteria, protozoa, fungi, nematodes, which can under the favourable conditions cause the massive insect mortality normally live in nature. Epizootics caused by viral and fungal pathogens such as well-known *Lymantria dispar* multicapsid nuclear polyhedrosis virus (*Ld*MNPV) and *Entomophaga maimaiga* Humber, Shimazu & Soper are often responsible for the spectacular supression of insect pest populations (Evans, 1986; McCoy et al., 1988).

MATERIALS AND METHODS

Beech forests

In Serbia beech stands covers an area of 660,400 hectares (29.3 percent of the total area covered by forests) and are found at the altitudes ranging from 100 to 1.700 meters. They account for 47.11 percents of the total areas covered by forests in Central Serbia (Banković et al., 2009).

Environmental factors such as temperature, humidity, wind, and rain may contribute to the initiation of *Ld*MNPV and *E. maimaiga* epizootics. Due to the maritime and humid microclimate, as well as due to the thick canopy closure of the tree layer, in the montane beech forests, microclimate conditions are very favourable - under the tree crowns the relative air humidity is high, and insolation and stronger air flows are minimal. The litter made of the beech and mixed species enable the formation of deep, moist and fertile eutric and dystric brown soils. Such microclimate conditions fulfill the necessities for development of *Ld*MNPV and *E. maimaiga* and enable them to achieve the state of epizootics in the beech stands and cause the collapse of the outbreak of the gypsy moths in the altered, unfavourable, global climate conditions of central Serbia over the period of research.

Gypsy moth *Lymantria dispar* L. (Lepidoptera: Erebidae)

The gypsy moth is one of the most dangerous pests of broadleaf forests and orchards. It is characterised by a high reproductive capacity, considerable ecological plasticity and polyphagia. It occurs periodically in high numbers (outbreak). Although it is found on four continents (North Africa, Asia, Europe, North America), the greatest damage is caused to the forests of the Balkan Peninsula, which have all favourable environmental conditions for the gypsy moth development, and it often occurs in outbreaks. During the progradation years of the latest outbreak (2010-2011, 2011-2012) the gypsy moth infested the forests of the central part of Republic of Serbia, the area of 9,021 ha and 30,380 ha. The culmination occurred in the periods 2012-2013 and 2013-2014, i.e. the infested territory covered an area of 184,444 ha and 339,987 ha, subsequently. At some sites the number of oviposited egg masses per unit area (ha) amounted to more than 50,000. The average number of eggs in an egg mass ranged from 504.8 (2011) to 585.3 (2013) (Tabaković-Tošić, 2013).

Lymantria dispar multicapsid nuclear polyhedrosis virus (Baculoviridae: Alphabaculovirus)

*Ld*MNPV is peculiar to the gypsy moth, one of the most devastating natural disease, and it causes a dramatic collapse of outbreak populations by killing both the larvae and pupae.

Transmission of *Ld*MNPV in an insect population occurs either via ingestion of food, via soil contaminated with occlusion bodies by insect larvae or via oviposition of eggs contaminated with occlusion bodies. Infection is initiated when alkali sensitive occlusion bodies are ingested by a susceptible host and dissolved by juices in the insect midgut. The dissolution is aided both by the high pH and the presence of alkaline proteinases in the midgut lumen. The virions released from occlusion-derived virus (ODV) then pass through the peritrophic membrane and fuse with the midgut epithelial cell plasma membrane. The nucleocapsids are subsequently released into the cytoplasm and migrate to the nucleus, where transcription of viral genes and replication of the viral genome take place. The nucleocapsids synthesized in the nucleus pass through the nuclear membrane and bud and acquire a new envelope from the plasmalemma to become budded virus (BV). The BVs produced in epithelial cells of the midgut spread via the hemolymph and/or tracheal system into all tissues of the insect causing a secondary infection, but the predominant target is the fat body cell. In the early stage of secondary infection, infected cells produce BVs which efficiently spread the infection from cell to cell within insect tissues. At later stage of secondary infection, virions are occluded into occlusion bodies in the infected cells. At the end of the infection, the cells and tissues of the dead insects are disintegrated and occlusion bodies are released into the environment. (Hu et al., 2002).

Entomophaga maimaiga Humber, Shimazu & Soper (Entomophtorales: Entomophtoraceae)

Entomopathogenic fungus E. maimaiga was isolated and described as a natural enemy of the gypsy moth in Japan, where it causes periodical epizooties. It is also spread in some parts of China and the Russian Far East (Hajek et al., 2005). In spite of the fact that

it was introduced in North America in 1910-1911, its presence in the natural populations of gypsy moth was determined only in 1989 (Hajek et al., 1996), when the pathogen caused pandemic in several countries (Andreadis and Weseloh, 1990; Smitley et al., 1996). Today *E. maimaiga* is a very significant pathogen of the gypsy moth in North America and Canada.

Bulgaria has been the second country in the world and the first one in Europe in which *E. maimaiga* was introduced successfully (Pilarska et al., 2006). For a period of 10-12 years, *E. maimaiga* expanded its range (naturally and by introductions) and is now found throughout Bulgaria (Mirchev et al., 2013). This entomopathogenic species has slowly spread along the Balkan Peninsula and Southeast Europe, and so far its presence has been reported also in European part of Turkey (Georgiev et al., 2012), Serbia (Tabakovic-Tosic et al., 2012, 2013), Greece, FYR Macedonia (Georgieva et al., 2013), Croatia (Hrašovec et al., 2013), Hungary (Csóka et al., 2014), Slovakia (Zúbrik et al., 2014), Bosnia and Herzegovina (Milotić et al., 2015). Regarding the situation in Serbia, *E. maimaiga* was first established to spread naturally in two regions in central part of the country, Belgrade and Valjevo (Tabaković-Tošić et al., 2012). Then, the fungus was introduced into *L. dispar* population situated in mountain Avala in Belgrade region and was also introduced or found in many places of the country (Tabaković-Tošić, 2014a, b).

E. maimaiga passes the winter as tough, thick-walled azygospores in the soil and on tree bark. In May or June, resting spores germinate and produce sticky spores at the end of a stalk that grows just above the soil surface. Gypsy moth caterpillars come into contact with these spores as they search for suitable leaves to feed on. The fungus digests its way through the exoskeleton of the caterpillar and grows inside the body of the caterpillar. Infected caterpillars may die within one week. When young caterpillars are affected early in the summer, the fungus will produce a second type of spore called conidiospores. These microscopic spores are spread by the wind and can infect other caterpillars.

From all sites, in the summer and autumn of the period 2010-2014, dead gypsy moth larvae were collected for different laboratory analyses. Each study plot was 1 ha in size and included a minimum of 25 trees. Gypsy moth dead caterpillars were collected manually from foliage in the lower parts of tree crowns and tree branches and trunk, two to three times per year.

Microscopic (magnification 1200 times) analyses of some dead gypsy moth larvae with characteristic symptoms of *Ld*MNPV was conducted immediately using the standard method of Giemsa's differential staining.

The dead larvae with characteristic symptoms caused by the entomopathogenic fungus *E. maimaiga* were also placed in Petri dishes and the detailed microscope survey of the dead gypsy moth caterpillars was done later. The evaluation of *E. maimaiga* infections was recorded as positive when azygospores and conidiospores were detected in the cadavers of dead gypsy moth larvae. The species identification was based on the size, shape and structural characteristics of different life forms of the fungus – azygospores, conidiospores and mycelia.

RESULTS

In the spring 2011, 2012, 2013 and 2014 selected beech forest areas in Central Serbia (Table 1), where a great increase of the population size of the gypsy moth was reported, were observed in a great detail. In many sites, it was first observed that there was no considerable damage of the foliage, which would normally be clearly visible. Even at the sample plots where the intensity of the attack was equal to several hundred egg masses per a hectare, which implies that the next instar can cause 100 percent defoliation, the trees looked as if the gypsy moth was in the progradation phase. In addition, at some sample plots the increased mortality rate of the younger and older larval instars in comparison with the expected one was reported. Logically, the following question was posed: What does prevent the larvae from intensive feeding and doing harm and what has been killing them? In order to get the answer to this question, detailed analysis of the possible causes was conducted.

The field studies conducted in May, June and July of 2011-2014 revealed a huge amount of dead larvae on the trees. Detailed study of the dead gypsy moth larvae showed two groups of the characteristic symptoms: first - caused by *E. maimaiga*, and second - caused by the *Ld*MNPV.

*Ld*MNPV was observed in 2.0-20.7 % of the dead larvae. Only in the studied beech stands in Kuršumlija gypsy moth caterpillars with clear symtomps of the viral diseases were not found (Table 1). In the studied area of central Serbia clear and characteric symtoms of the fungal diseases were detected in 79.3-100% of the reported dead gypsy moth larvae (Table 1).

Table 1. Gypsy moth larvae mortality analysis.

Locality - Region	Year of the sampling	Gypsy moth attack intensity*	Larvae with symptoms caused by	
			Entomophaga maimaiga (%)	Baculovirus LdMNPV (%)
Beograd	2011	Severe	85.0	15.0
Valjevo - Bogovadja	2011	severe	92.4	7.6
Krupanj	2013	severe	94.1	5.9
Kučevo	2013	Severe	87.3	12.7
Majdanpek	2014	severe	79.3	20.7
Kragujevac	2013	medium	93.4	6.6
	2014	severe	81,8	18,2
Kraljevo	2013	medium	96.7	3.3
Kruševac	2013	Severe	84,2	15,8
	2014	high	98.0	2.0
Vrnjačka Banja	2013	high	96.2	3.8
Brus	2013	high	88.7	11.3
Blace	2013	high	82.9	17.1
Kuršumlija	2014	severe	100	0
Prokuplje	2014	severe	89,1	10,9
Donji Milanovac	2012	severe	94.5	5.5
	2013	high	86,5	13,5
	2014	high	79,9	20,1
Negotin	2012	severe	94.0	6.0
Leskovac	2013	medium	98.0	2.0
	2014	severe	85,3	14,7
Vučje	2014	severe	83,1	16,9
Sokobanja	2014	severe	87,6	12,4

*11-100 egg masses/hectare - medium, 101-500 – high, and over 500 - severe.

The presence of *Ld*MNPV occlusion bodies or *E. maimaiga* conidia/azigospores was confirmed in the dead larvae, but the presence of *Ld*MNPV was reported only in the older caterpillars (L_4-L_6). It should be noted that a higher number of the infected larvae was presented in areas where the intensity of the attack of the gypsy moths was very strong and where there were several thousand of the egg masses per hectare (Table 1). In the beech forests of the studied area, the collapse of the gypsy moth outbreak was caused by *E. maimaiga* (Table 1). Presence of a higher number of conidiospores (in younger larval instars) and azygospores (dominant in the older larval instars of gypsy moths) was clearly determined.

The reason of such occurrence of two studied pathogens lies in the fact that the fungus is active at low, as well as high, gypsy moth population levels. This sets it apart from natural biocontrol agents, such as the *Ld*MNPV, which kills caterpillars when the population densities are high and the food supplies is unsufficient. In the literature there are data showing slow negative impact of *E. maimaiga* on *Ld*MNPV or synergistic relation between them (Malakar et al., 1999).

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THE DEVELOPMENT OF GYPSY MOTH (*Lymantria dispar* L.) UNDER DIFFERENT TEMPERATURES

Rudolf Hillebrand, Katalin Tuba and Ferenc Lakatos

University of West-Hungary
Institute of Silviculture and Forest Protection
H-9401 Sopron Pf. 132
hillebrand.rudolf@emk.nyme.hu

ABSTRACT

The gypsy moth is one of the most important defoliator moth species. The size of the damaged areas can be huge, especially during its periodic outbreaks. The appearance of this species is recorded for a long while and its developmental life stages plays dominant part in numerous researches. The outbreak of the gypsy moth strongly depends on the weather conditions. But the unfavorable environmental conditions can weaken not only the health of forests, but the dry years or uneven rainfalls can also affect the development of the gypsy moth. If the average annual temperature is increasing because of the climate change, does it have effect on the individuals of the species as well? In our trial the development of 3 samples were investigated on three different temperatures (20 °C, 25 °C, 30 °C) under laboratory conditions. Each sample consists of thirty larvae. We raised the caterpillars feeding on artificial food, on each temperature. During the investigation we measured and compared the developmental periods, the weights, the number of larval stages, the mortality and the fecundity. Our results show, that the temperature has significant impact on the developmental characteristic and the fecundity of this pest as well as its damages.

INTRODUCTION

The gypsy moth (*Lymantria dispar*, Linnaeus 1758) is one of the most important, harmful, herbivore species is in the deciduous forests. The damaged areas can be huge, especially during its periodic outbreaks. It impaired more than 200 000 hectare big area in Hungary during its last outbreak (2003-2006) (Csóka and Hirka, 2009). The appearance of this species has been recorded for a long while and its life cycle is the subject of plenty of researches. The outbreaks and damages of the gypsy moth are strongly influenced by the weather conditions. The unfavourable environmental conditions can weaken not only the health of forests, but dry years or uneven rainfalls can also influence in a negative or a positive way the development of the gypsy moth (Csóka, 1996).

Our aims were to investigate the effect of the temperature on the life cycle and on the development of gypsy moth. This is an important question regarding the procession of the climate changing.

MATERIAL AND METHODS

The samples (egg masses) were collected in Hegyesd in Hungary. The original food plants of the moths were pedunculate oak (*Quercus robur* L.), Turkey oak (*Quercus cerris* L.) and manna ash (*Fraxinus ornus* L.).

Conditions of the experiment:

- Light and darkness:	16 : 8
- Food:	artificial food
- Temperature:	20 °C; 25 °C; 30 °C
- Number of the samples:	30-30 individuals on three different temperatures.

Course of the experiment:

- We collected egg masses.
- The eggs were kept in fridge until the beginning of the experiment.
- They were placed into plastic boxes and were hatched under laboratory conditions.
- The larvae were reared until pupation.
- The moths were crossed.

Measured data:

- The date
  - of the larva-hatching,
  - of the molt,
  - of the pupation,
  - of the moth-hatching.
- Weight of the larvae (0,000 g).

RESULTS

The rearing of the 30-30 gypsy moth larvae was successful on three different temperatures. The mortality was very low. Two individuals died on 20 °C, and three individuals ruined on 30 °C. There wasn't any mortality on 25 °C. The data of development time is represented in the first table.

The differences are proved by these data and the first and second figures well present them, too.

The data of Larva-weight-growth was presented by the second table.

These data show the difference between male and female larvae. The third and fourth figures illustrate the larval weight and growth on three different temperatures.

Table 1. Development time (day)

Sex	Temp. (°C)	Mean					SD	Max.	Min.
		L_4	L_5	L_6	L_7	L_4-pupa	L_4-pupa.	L_4-pupa	L_4-pupa
male	20	11	18	14		30	3.1575	37	25
	25	7	12	12		21	3.9812	32	16
	30	7	10			17	3.1990	21	12
female	20	10	10	16	19	42	7.7782	57	29
	25	6	7	12	16	28	5.3847	41	22
	30	5	7	11	13	23	5.0448	33	14

Legend: Temp.: temperature; L_4-pupa: development time between L_4 and pupation

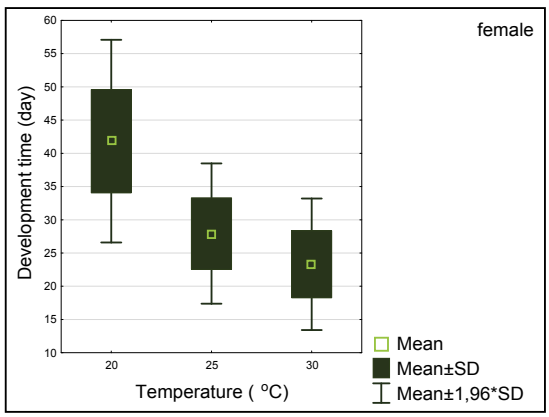

Figure 1. Development time (L4-pupa) influenced by temperature (female)

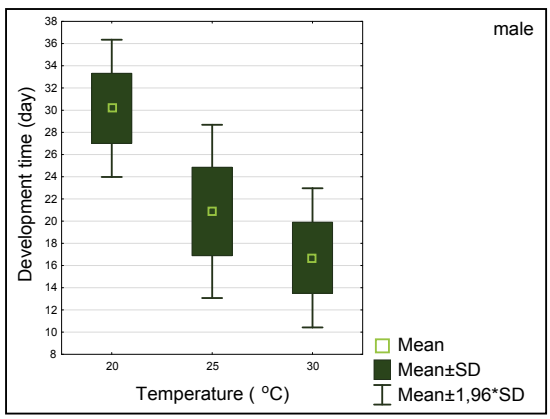

Figure 2. Development time (L4-pupa) influenced by temperature (male)

Table 2. Larva-weight-growth (g)

Sex	Temp. (°C)	Mean					SD	Max.	Min.
		L_4	L_5	L_6	L_7	L_4-pupa	L_4-pupa.	L_4-pupa	L_4-pupa
male	20	0.0855	0.2682	0.2493		0.3745	0.1025	0.5865	0.2537
	25	0.0912	0.2558	0.291		0.4015	0.0966	0.5637	0.2745
	30	0.1328	0.1935			0.3263	0.1299	0.5516	0.0535
female	20	0.0814	0.1987	0.7081	0.8764	1.2803	0.5884	2.676	0.2887
	25	0.0806	0.2033	1.0307	1.3792	1.5116	0.4279	2.401	0.869
	30	0.0934	0.2487	0.5398	0.6529	0.9163	0.2352	1.4848	0.4283

Legend: Temp.: temperature; L_4-pupa: larva-weight-growth between L4 and pupation

We used the Kruskal-Wallis test. We could prove the differences among gypsy moth larvae reared on the three different temperatures. The test pointed at the significant differences (3. and 4. tables). The tables don't show any results about L7 larval stages because only few individuals reached this larval stage so these data weren't comparable.

DISCUSSION

There were significant differences among the development time of the gypsy moth under different temperatures. The temperature influences the development of the larvae in a serious way.

- The development of the gypsy moth is shorter in 30 °C, than the other two temperatures (20 °C, 25 °C). Significant differences were recorded between 20 °C and 25 °C and 20 °C and 30 °C regarding the development periods.

- The temperature strongly influences the larva weight growth, too. However significant differences were found only in the 6th larval stage and the whole development of females. So the females react more sensible to the temperature.

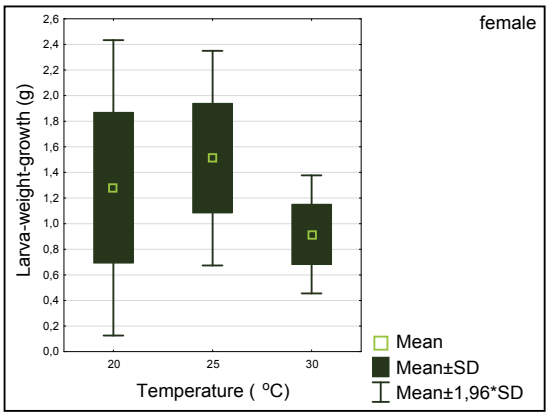

Figure 3. Larva-weight-growth influenced by temperature (L_4-pupa) (female)

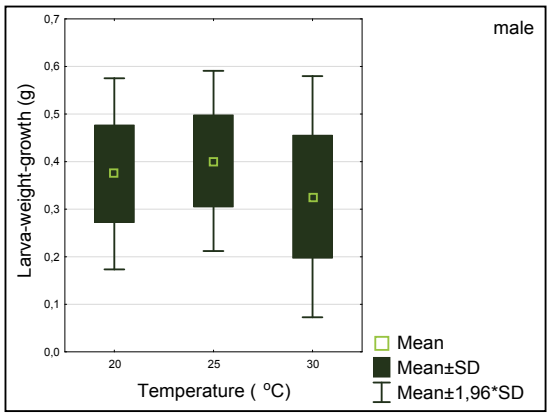

Figure 4. Larva-weight-growth influenced by temperature (L_4-pupa) (male)

Table 3. Development time (p value)

Sex		L_4				L_5		
male	Temperature	20 (°C)	25 (°C)	30 (°C)	Temperature	20 (°C)	25 (°C)	30 (°C)
	20 (°C)		0.000772	0.000945	20 (°C)		0.005209	0.000154
	25 (°C)	0.000772		1	25 (°C)	0.005209		0.546847
	30 (°C)	0.000945	1		30 (°C)	0.000154	0.546847	
female	Temperature	20 (°C)	25 (°C)	30 (°C)	Temperature	20 (°C)	25 (°C)	30 (°C)
	20 (°C)		0.01494	0	20 (°C)		0.001061	0.005322
	25 (°C)	0.01494		0.096479	25 (°C)	0.001061		1
	30 (°C)	0	0.096478		30 (°C)	0.005322	1	
Sex		L_6				L_4-pupa		
male	Temperature	20 (°C)	25 (°C)	30 (°C)	Temperature	20 (°C)	25 (°C)	30 (°C)
	20 (°C)				20 (°C)		0.001868	0.000006
	25 (°C)	The number of samples is low.			25 (°C)	0.001868		0.205337
	30 (°C)				30 (°C)	0.000006	0.205337	
female	Temperature	20 (°C)	25 (°C)	30 (°C)	Temperature	20 (°C)	25 (°C)	30 (°C)
	20 (°C)		0.337774	0.020911	20 (°C)		0.001317	0
	25 (°C)	0.337774		1	25 (°C)	0.001317		0.245577
	30 (°C)	0.020911	1		30 (°C)	0	0.245577	

Legend: L_4-pupa: development time between L_4 and pupation

Table 4. Larva-weight-growth (p value)

Sex		L_4				L_5		
male	Temperature	20 (°C)	25 (°C)	30 (°C)	Temperature	20 (°C)	25 (°C)	30 (°C)
	20 (°C)		1	0.711452	20 (°C)		1	0.212119
	25 (°C)	1		0.569130	25 (°C)	1		0.085187
	30 (°C)	0.711452	0.56913		30 (°C)	0.212119	0.085187	
female	Temperature	20 (°C)	25 (°C)	30 (°C)	Temperature	20 (°C)	25 (°C)	30 (°C)
	20 (°C)		1	0.430673	20 (°C)		1	0.24175
	25 (°C)	1		0.679091	25 (°C)	1		0.380192
	30 (°C)	0.430673	0.679091		30 (°C)	0.241750	0.380192	
Sex		L_6				L_4-pupa		
male	Temperature	20 (°C)	25 (°C)	30 (°C)	Temperature	20 (°C)	25 (°C)	30 (°C)
	20 (°C)				20 (°C)		1	1
	25 (°C)	The number of samples is low.			25 (°C)	1		0.49653
	30 (°C)				30 (°C)	1	0.49653	
female	Temperature	20 (°C)	25 (°C)	30 (°C)	Temperature	20 (°C)	25 (°C)	30 (°C)
	20 (°C)		0.092585	0.877776	20 (°C)		0.377219	0.033863
	25 (°C)	0.092585		0.004621	25 (°C)	0.377219		0.000271
	30 (°C)	0.877776	0.004621		30 (°C)	0.033863	0.000271	
Legend:	L_4-pupa:	larva-weight-growth between L_4 and pupation						

- Regarding my earlier experiments there is a serious difference between the development of the male and the female gypsy moth (development time, larva weight growth, leaf consumption, different nutritional indices (Hillebrand and Tuba, 2013).

- The 25 °C affected the most favourable the growth of the larvae. The development time was by far shorter in higher temperature. According to the development of the gypsy moth the optimal temperature is about 25 °C.

- The gypsy moth causes huge damage in its larval stages. The lengths of the development time influence the rate of the damage. The larva weight growth corresponds with the efficacy of the feeding and amount of the leaves consumed. The temperature influences both the development period and the growth of the larva weight of the gypsy moth so the temperature has effect on its damage level.

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COLOUR AND SEX RATIOS IN DIFFERENT BOX TREE MOTH (*Cydalima perspectalis*) POPULATIONS

Katalin Tuba, Géza Kelemen and Miklós Molnár

University of West-Hungary
Institute of Silviculture and Forest Protection
H-9401 Sopron Pf. 132
tuba.katalin@emk.nyme.hu

ABSTRACT

The box tree moth, *Cydalima perspectalis* (Walker 1859), is native to East Asia and has recently been introduced in Europe. It was first recorded in South Germany in 2007. This alien moth has been causing severe damage to topiary box tree, hedges and plants in parks, gardens, cemeteries and nurseries as well as to native populations of the box-tree (*Buxus* spp.) in Europe. The larvae feed on leaves but they can also attack the bark. The affected bushes die after repeated infestations. The box tree moth is a polymorph species. Our aim was to investigate the sex ratio and this polymorphism regarding the appearance of the colours in the case of the sexes.

To reach these goals to use of both field and laboratory techniques were required. The number and distribution of morphs were studied in different ways. 1) Light trap data were analysed in 2013 and 2014. 2) The data received from EDDMapS were also gathered and grouped to estimate the colour and sex ratios. 3) More than 100 larvae or pupae were collected from different sites in 2013-2014. These specimens were reared under laboratory conditions. The adults were examined and classified. 4) Last but not least we also gained genetic data from crossings.

Three colour morphs were divided in the Hungarian *C. perspectalis* populations. The white morphs with brown band on the exterior margin only and white ones with brown band both on the exterior and interior margins were observed to start almost flying together. The dark ones fly the latest and the shortest time. The first generation flies for a short time while the third one flies for the longest time. The number of males is usually higher in the population than the number of females. Our results show that the ratio of the three morphotypes varied in different habitats. It seems that the white morph appears first during the course of spread. The crossings resulted in a special ratio among the morphs.

Keywords: alien species, morphotypes, distribution

INTRODUCTION

The box tree moth is a relatively new alien species in Europe. It is native to East Asia (Inoue et al., 1982). Its native range here is determined by the occurrence of its hosts and the climatic conditions. The limit of its spread is the isotherm -16 °C in winter. So it might spread across most of Europe, except for North Fenno-Scandinavia, northern Scotland and high mountain regions, where the accumulated temperatures are not enough to allow the completion of an entire generation a year (Nacambo et al., 2013).

Cydalima perspectalis was first detected in south-western Germany in Europe in 2007, but it had presumably arrived in Germany a bit earlier because the infection observed was very high and extended (Billen, 2007; Krüger, 2008; Leuthardt et al., 2010). Soon it was recorded in Switzerland (Leuthardt et al., 2010). The box tree moth was found in the Netherlands (Muus et al., 2009) in 2007; in France (Feldtrauer et al., 2009), in Britain (Mitchell, 2009), in *Liechtenstein (*Slamka, 2010) in 2008*;* in Austria (Rodeland, 2009) in 2009; in Belgium (Casteels et al. 2011, De Prins & Steeman, 2011), in Italy (Biondi, 2010), in Slovenia (Jež, 2012), in Hungary (Sáfián & Horváth, 2011), in the Czech Republic (Šumpich, 2011), in Romania (Székely et al., 2011), in Turkey (Hizal et al., 2012) in 2011; in Slovakia (Pastorális et al., 2013) and in Croatia (Koren & Črne, 2012), in Sochi in 2012; in Denmark in 2013 (Rennwald, 2015).

Main hosts of the box tree moth are different boxwood species in Europe (CABI, 2013). *C. perspectalis* larvae have been observed as causing feeding damage by chewing the leaves and tree-bark of these plants. Complete defoliation causes the mortality of the damaged boxwood (Albert & Lehneis 2012; Kenis et al. 2013; Leuthardt & Baur 2013; Nacambo et al. 2013).There are two *Buxus* species, which are native to Europe: *B. sempervirens* and *B. balearica (*Di Domenico et al., 2012). However, in Asia, other reported hosts include *Ilex purpurea*, *Euonymus japonicas, Euonymus alatus, Pachysandra terminalis* and *Murraya paniculata* (Korycinska & Eyre, 2011; Wang, 2008). The natural populations of *Buxus* are seriously threatened by *C. perspectalis* (Kenis et al. 2013). Cultivated boxwood plants in parks, cemeteries and gardens are also damaged by this pest under urban conditions.

There are several species that have morphotypes. The morphological differences can appear in different developmental stages. The causes of these phenomena originate under different conditions. Two sympatric colour morphs of *Zeiraphera diniana* were described that are distinguishable only during the final larval stage; the dark one is found mainly on *Larix decidua* and the orange-yellow morph occurs on *Pinus cembra* (Bovey & Maksymov, 1959). Larvae of *Zeiraphera diniana* were raised continuously or at least during their latter larval stage at 10 °C were darker than larvae reared at 18 °C (Baltensweiler, 1977). Nutritional stress causes an increase in frequency of intermediate morphotypes in subsequent generations (Day & Baltensweiler, 1972).

Two or more morphs may be recognised e.g. in the cases of *Odontopera bidentata* (Kettlewe, 1959; 1973), *Biston betularius* (Clarke & Sheppard 1964; Lees, 1968) and *Phigalia pilosaria* (Lees, 1974), *Allophyes oxyacanthae* (Steward, 1977), and *Simyra albovenosa* (Vakkari, 1980). The increase in the proportion of dark *Biston betularius* morphotype appears to be a phenomenon for which no explanation has yet been presented in the scientific literature. Bishop and Cook (1980) say:"The reason is not obvious". The melanic phenotype is inherited as autosomal dominants (Grant, 2004). Two melanic alleles are present regarding *Phigalia pedaria* moth but also occur at combined frequencies of 2-18% in rural areas across the British Isles (Lees 1971). Steward (1977) says that selective predation could be a major factor in the variation of melanic frequencies of *Diurnea fagella* and *Allophyes oxyacanthae*. The uniform black and the patterned intermediate form of *Simyra albovenosa* are controlled by separate loci. The dark one is completely dominant to pale and epistatic to intermediate, and the patterned one is partially dominant to pale. The intensity of the whitish to yellowish background colouration is affected by the developing strategy. Non-diapausing moths are paler than diapausing (Vakkari, 1980).

MATERIAL AND METHODS

The number and distribution of morphs were observed in different ways.

1) Light trap data were analysed from the beginning of May until the end of October 2013 and 2014. The light trap was situated in Sopron where the first *C. perspectalis* was caught in Hungary in 2011. The different sexes and colours were separated each day.

2) Photos of the moth received from EDDMapS were also gathered and grouped to estimate the ratios of the colours and sexes. EDDMapS is an Early Detection and Distribution Mapping System, which has been operated by Bugwood Network since 2008. Our institute has adopted this system and adjusted to the Hungarian conditions. Spreading data of *C. perspectalis* have been collected with this system in Hungary since March 2012.

3) One hundred larvae were collected in Gyöngyösfalu and Sopron in May 2013, and one hundred pupae were gathered in Kőszeg and Pápa, Hungary in June 2014. These specimens were reared under laboratory conditions (20 °C, 16L:8D photoperiod). The larvae were fed with leaves of boxwood. The adults were examined and classified based on colour and sex.

4) Last but not least we also gained data from crossings. This survey was carried out in a period of two years. Four pairs of the moth were placed in a 40x25x25 cm box, and

they were fed with a mixed solution of honey and water. The larvae hatched were placed into a 0.5 l plastic box. The rearing method was similar to that in the case of larvae and pupae (3). Regarding this examination the white with brown band on the exterior margin only and the white with brown band both on the exterior and interior margins morphs were handled together, because it was not possible to do proper crossings among the three morphs neither next to high number of population in same time.

RESULTS

In Hungary, three colour morphs were observed. 1) The white with brown band on the exterior margin only (hereafter white) 2) the white with brown band both on the exterior and interior margins (of the forewing) (hereafter white with brown margin) and 3) the melanic (greyish-brown) form.

1) Light trap data

The catches of the light trap show similarity in colours and between years. The number of females was a slightly higher than the number of males. Considering the females the melanic form occurred in the highest ratio, while the white with brown margin form was the most frequent among the males (Tab. 1).

The flight periods of the three colour morphs were variable but partly overlapping. The white and white with brown margin morphotypes have a bit longer flight periods. The melanic form starts to fly later than the other two forms (Fig. 1).

Table 1. Colour and sex ratios of *C. perspectalis* morphotypes based on catches of the light trap

Male			Female		
White	White with brown margin	Greyish-brown	White	White with brown margin	Greyish-brown
2013					
10.1	19.4	17.0	13.2	19.3	21.0
2014					
8.5	20.3	13.6	11.9	22.0	23.7

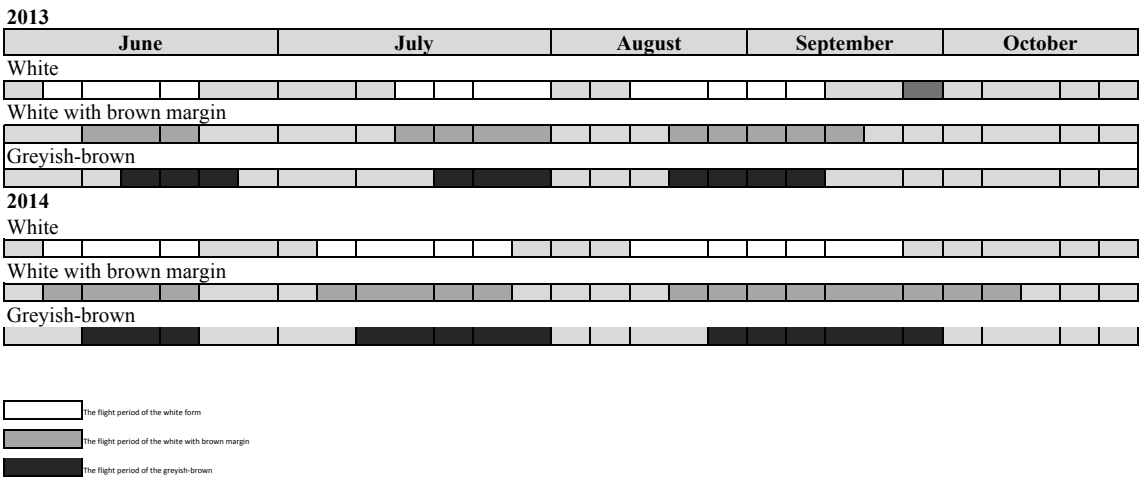

Figure 1. The flight periods of the morphotypes of *C. perspectalis*

2) EDDMapS data

EDDMapS data show that the white morph was the dominant in comparison of the recognised specimens (Fig. 2). The results regarding the data of the observations suggested that the first moths emerge between the white morph.

3) Results of rearing

There were not big differences between the two sexes regarding the ratios of the colours. In Sopron, where *C. perspectalis* was recognised in September 2011 and the sample of larvae were collected in May 2013 the melanic morph was the most frequent. In Kőszeg, where the moths were found in June 2013 and the sample was gathered in July 2014 the white with brown margin reached the highest number. In Gyöngyösfalu, where the larvae were found in Augustus 2013 and in Pápa, where the larvae were found in Augustus 2014 and the samples were collected directly after the finding the most frequent morph was the white type (Fig. 3, Fig. 4).

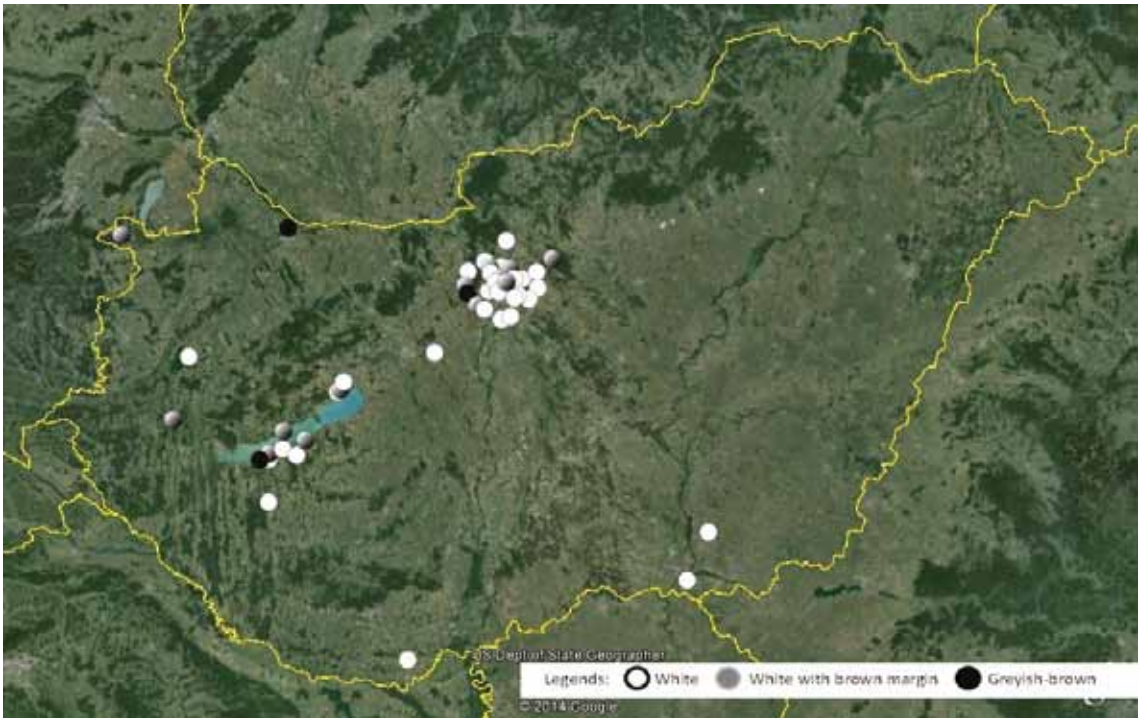

Figure 2. Occurrence of the three morphs of *C. perspectalis* based on public notifications

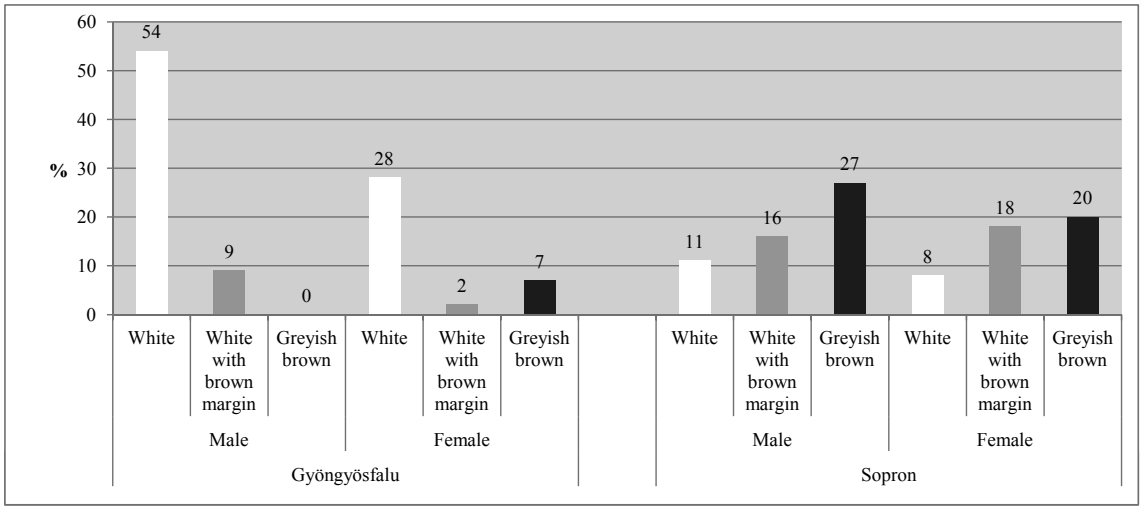

Figure 3. Colours and sex ratios of the reared *C. perspectalis* larvae

4) Results of crossing

The results of the crossing indicated a special ratio between the white and white with brown margin together and greyish-brown colour morphs in both years. We can also find a similarly special ratio by analysing the relationship between the sexes (Fig. 5).

DISCUSSION

The white and the white with brown margin morphs of *C. perspectalis* were found to start to fly earlier than the dark ones. The first generation flies for a short time while the third one flies for the longest time. The males of the box tree moth hatched earlier than the females. The white morph appears first during the course of spread.

Our result shows that the ratio of the three morphotypes was different at different locations. It seems that the most frequent colour form may correlate with the length of the period after the ecesis or with the number of generations (1st, 2nd or 3rd). Directly after ecesis the white morph dominates in the habitats, while one year later (3-5 generations) the white with brown form reach the highest number, while later (6-9 generations) the melanic form is present in the highest number. The other explanation is that the melanic morphs reach a higher number in the first generation, the white with brown margin morphs reach a higher number in the second generation while in the third generation the white form can be found in the highest number. This phenomenon may correlate with the temperature or the length of the day. However, further examinations are needed to answer this question.

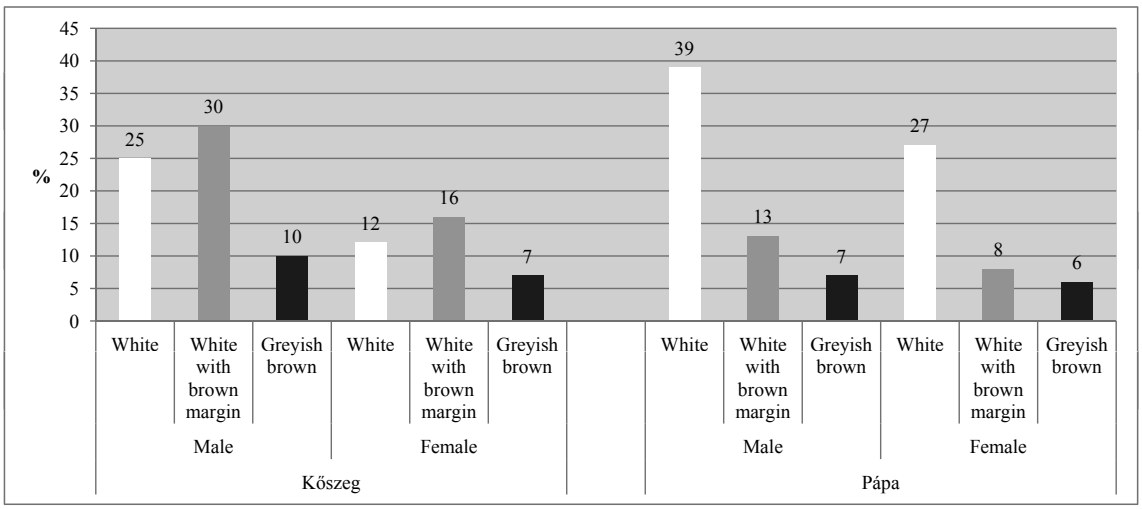

Figure 4. Colours and sex ratios of the reared *C. perspectalis* pupae

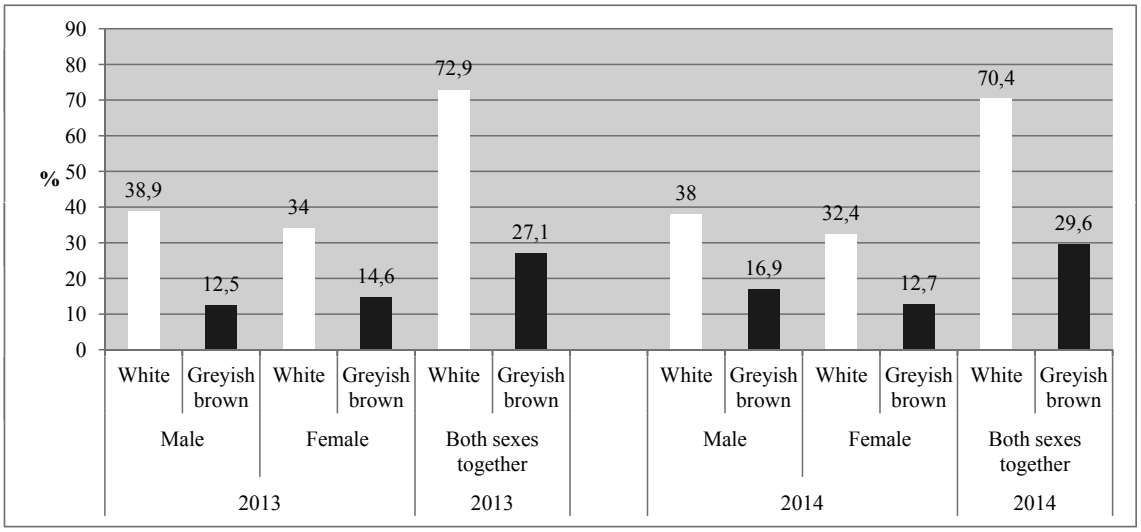

Figure 5. Results of the *C. perspectalis* crossing experiment

The crossings resulted in a special ratio between the white and white with brown margin together and melanic greyish-brown morphs in both years. Regarding the results of rearing and crossings the number of males is higher in the population than the number of females. Korycinska and Eyre (2011) say there are two morphotypes, a white one and a greyish-brown one. Based on our results it is suggested to divide the population of *C. perspectalis* into three morphotypes: 1) the white with brown band on the exterior margin only 2) the white with brown band both on the exterior and interior margins (of the forewing) and 3) the melanic form.

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PINE WOOD NEMATODE *Bursaphelenchus xylophilus* SURVEY IN CONIFEROUS FORESTS IN SERBIA

Jasmina Bačić[1], Barbara Gerič Stare[2], Gregor Urek[2] and Saša Širca[2]

[1] Agricultural Extension Service Institute Tamiš, Novoseljanski put 33, 26000 Pančevo, Serbia
[2] Agricultural Institute of Slovenia, Hacquetova 17, 1001 Ljubljana, Slovenia
E-mail: jasmina.bacic@jasminabacic.rs

ABSTRACT

An official survey to detect the quarantine pine wood nematode *Bursaphelenchus xylophilus* was conducted in 2012-2013 in coniferous forests in Serbia. In total, 55 samples of wilted and cut trees were examined. According to the results of the two-year monitoring, *B. xylophilus* has not been found in Serbia. A morphologically very close species *Bursaphelenchus mucronatus kolymensis* also referred as the European type of *B. mucronatus* was detected in a sample from a forty year dying *Pinus sylvestris* tree from mountain of Divčibare in western Serbia. Morphological identification was confirmed by molecular method ITS-RFLP. Detailed measurements of *B. mucronatus kolymensis* detected for the first time in Serbia are presented.

Key words: pine wood nematode, *Bursaphelenchus*, morphological characters, Serbia

INTRODUCTION

Pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, a causal agent of pine wilt disease is worldwide threat to forest ecosystems. This species is included on the list of EPPO A2 quarantine organisms and recommended for regulation by Directive 2000/29/EC in the European Union. Special phytosanitary measures apply against spread of this pest as well as monitoring of the PWN presence in the coniferous forests in EU countries since its first report in Europe, in Portugal in 1999 (Mota et al., 1999) and more recently in Spain (Abelleira et al., 2011). It represents a potential threat to coniferous forests especially in southern area of Europe, including region of Serbia due climatic conditions with high summer temperatures that are suitable for establishment of this pest (Evans et al., 2008). The second risk factor is the presence of vector insects of the genus *Monochamus* in Serbia (Coleoptera: *Cerambycidae*): *M. galloprovincialis*, *M. sutor*, *M. sartor* and *M. saltuarius* (Ilić, 2005).

According to Banković et al. (2009), the total forest area in Serbia consists of 2.252.400 ha (29,1 % of the total land area of the country) of which 12,3 % are the conifers, among which three species are dominant: *Picea abies* (2,7 %), *Pinus nigra* and *P. sylvestris* (3,8 %). In 2011, Serbian Ministry of Agriculture, Forestry and Water Management introduced legislation on implementation of phytosanitary measures for detection of this harmful organism and measures to be undertaken in case of its occurrence in the territory of the Republic of Serbia. Prior to this survey, it was not known if *Bursaphelenchus* species were present in Serbia. The national monitoring aimed to determine the potential presence of PWN and occurrence of species belonging to *Bursaphelenchus* genus in Serbia.

MATERIAL AND METHODS

Sampling and nematode extraction

Official surveillance on the PWN presence in coniferous forests started in 2012 in the framework of implementation of the Programme of the annual

plant health protection measures of Serbian Plant Protection Directorate. Wood samples were collected from conifer forests during the summers of 2012-2013 from 9 forest administrations of State Enterprises for Forest Management "Srbijašume" and "Vojvodinašume". Wood samples were collected on suspicious trees showing fungal infection and insects holes by axe and low-speed drill (Φ13 mm). In total 55 samples of wilted and cut pine trees were collected (*Pinus sylvestris*, *P. nigra*, *Picea abies*, *Pseudotsuga menziesii*). Wood samples were placed into plastic bags and incubated at approximately +25°C for 14 days prior to nematode extraction by Baermann funnels. After 48 hours water was removed and extracted nematode suspensions were observed using a binocular stereomicroscope (EPPO, 2013). The nematodes belonging to the *Bursaphelenchus* genus were hand-picked from the suspension, heat killed at 65°C and mounted on temporary slides. The specimens were examined for morphological identification using a light microscopy Leica DM 1000 LED connected to a DFC 295 digital camera. Ten males and ten females were analysed with Leica Application Suite by interactive measurements. The following diagnostic characters were measured: body length and width, stylet length, tail length and width, vulva position (%), body length divided by width at mid-body (a), body length divided by tail length (c), tail length divided by body width at anus (c'), and spicules length measured along curved median line.

Molecular characterization

Morphological identification was confirmed by molecular method PCR-ITS-RFLP using primers 5'-CGTAACAAGGTAGCTGTAG-3' and 5'-TTTCACTCGCCGTTACTAAGG-3', and restriction enzymes *Rsa*I, *Hae*III, *Msp*I, *Hinf*I and *Alu*I (Burgermeister et al. 2009). DNA was isolated from one female and one male nematode separately. Restricted fragments were submitted to agarose gel electrophoresis and capillary electrophoresis system to comparatively test the method performance.

RESULTS

According to this two year survey, *B. xylophilus* has not been detected in Serbia. However, a morphologically very similar species *B. mucronatus kolymensis* also referred as the European type of *B. mucronatus* was found in a sample from a forty year dying *Pinus sylvestris* tree from mountain of Divčibare (Bačić et al., 2014). The tree infested with *B. mucronatus kolymensis* had visible signs of *Scolytidae* beetle attack. The morphological characters of *B. mucronatus kolymensis* are shown in **Table 1**.

Table 1. Measurements of *B. mucronatus kolymensis*, population from Serbia (Divčibare). Measurements in μm and in form: mean ± s.d. (range).

Characters	female	male
n	10	10
Stylet	14.0 ± 0.9 (12.4-15.2)	13.5 ± 0.7 (12.5-14.7)
Body length	781.0 ± 132.3 (601.8-1026.6)	741.2 ± 78.0 (610.7-878.1)
V %	71.0 ± 1.9 (68.3-75.5)	
Body width	17.9 ± 0.8 (16.0-18.8)	17.6 ± 1.0 (16.3-19.5)
Tail length	28.6 ± 3.2 (23.7-32.5)	31.8 ± 2.3 (27.7-35.0)
Tail width	9.4 ± 0.5 (8.5-10.3)	11.9 ± 0.8 (10.7-13.4)
a	43.5 ± 6.4 (32.1-55.2)	42.0 ± 3.9 (34.1-48.0)
c	31.9 ± 4.3 (27.8-31.9)	23.7 ± 3.5 (18.9-29.6)
c'	3.0 ± 0.3 (2.7-3.4)	2.6 ± 0.3 (2.2-3.1)
Spicule		26.6 ± 1.6 (23.1-28.1)

Nematode specimens had the following morphological characters: female - body ventrally arcuate after heat killing, stylet with small basal thickenings, vulva posterior with prominent cuticular flap, sub-cylindrical tail, mucro digitate, well offset from tail, tail mucro 3-4 μm long. Body of male J sharped when heat-killed, spicules paired, arcuate, cuculus disc-like, tail ventrally arcuate with bursa. The morphology of examined specimens corresponded to the descriptions of *B. mucronatus* European type (Braash, 2008).

Two electrophoresis methods were compared and both resulted in the identical RFLP patterns (**Figure 1**). Results of both analytical methods were in compliance with reported data in the literature (Burgermeister et al. 2009) and support the identification of the sample as *Bursaphelenchus mucronatus* European type = *B. mucronatus kolymensis*.

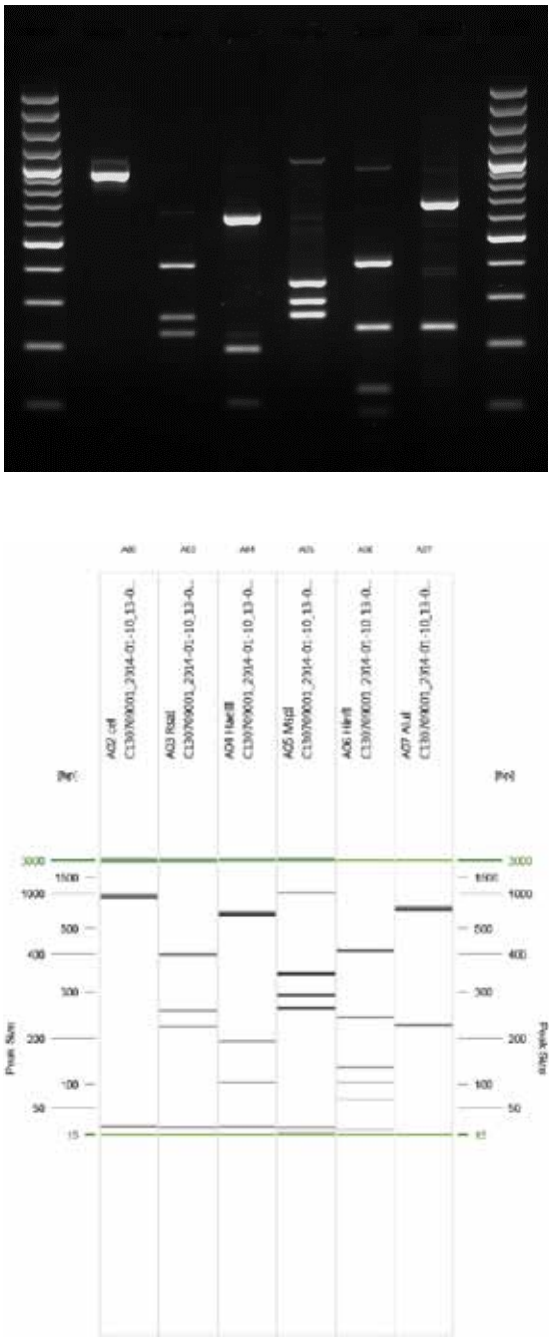

Figure 1. A: ITS-RFLP pattern of *B. mucronatus kolymensis* obtained on 1.5% agarose gel, from left to right: DNA marker 100 bp ladder Fermentas, with lowest band at 100 bp and prominent bands at 500 and 1000 bp; uncleaved PCR product; restriction fragments obtained with *Rsa*I, *Hae*III, *Msp*I, *Hinf*I, *Alu*I; and DNA marker. **B**: ITS-RFLP pattern of *B. mucronatus kolymensis* obtained with capillary electrophoresis, from left to right: uncleaved PCR product; restriction fragments obtained with *Rsa*I, *Hae*III, *Msp*I, *Hinf*I and *Alu*I.

DISCUSSION

Despite the absence of PWN in Serbia, obtained results of the monitoring programme between 2012 and 2013 manifested the occurrence of *Bursaphelenchus* species in the country for the first time. Clarifying the differences between the closely related species *B. mucronatus* and devastating *B. xylophilus* is essential in order to detect the possible presence of the pest. The main morphological difference between the two species is the shape of the female tail mucro (Mamiya & Enda, 1979). The females of *B. mucronatus* have mucronate tail end whereas *B. xylophilus* females are round-tailed. However, some populations of PWN vary in mucro shape which can lead to misidentification. The specific morphometric evidence for the European type subspecies of *B. mucronatus* was sub-cylindrical female tail with mucro 3-5 µm long, digitate, well offset from tail, whereas the East Asian type female tail is conoid, mucro pointed and almost continually with tail, length of mucro 4-7 µm (Braash, 2008). Since there is molecular evidence that even *B. xylophilus* and *B. mucronatus* may cross, actual or potential hybridization between populations of the two subspecies of *B. mucronatus* in nature cannot be excluded (Braash & Burgermeister, 2011). The European type is the most common in the northern coniferous forests extending from Scandinavia in the west to Kamtchatka in the east. It occurs in Central, East and South Europe and Turkey. The main vectors of this species in Europe are *Monochamus galloprovincialis* and *M. sutor.* The coniferous host species of the European type in Europe and Siberia are mainly *Pinus sylvestris*, *P. pinaster* and *Picea abies* (Braash & Burgermeister, 2011). The presence of *B. mucronatus kolymensis*, *Monochamus* vector species and suitable climatic conditions, increase the likelihood of *B. xylophilus* establishment and spreading in Serbia. Various studies have shown that in spite of quarantine regulations such as heat treatment, a global spreading of *Bursaphelenchus* spp. is still going on (Braash & Burgermeister, 2011). For that reason continuous surveillance and more intensive sampling of conifer forests should be conducted in the future in Serbia.

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ANALYSIS OF MECHANICAL STABILITY OF SOLITARY TREES

Géza Kelemen and Katalin Tuba

University of West Hungary
Institute of Silviculture and Forest Protection
H-9400 Sopron, P.o.box: 132. Hungary
ge.kelemen@gmail.com

ABSTRACT

This paper studies the specific circumstances of wind throw of solitary ornamental trees and presents typical examples using a relevantly and cheap method of mechanical calculations.

The work schematizes the forces causing windfall, the mechanical properties of trees by windfall and the givens of the environment of the trees. Understanding of the causes of stability loss in trees and the process of windfall is important for preventing accidents caused by wind thrown solitary trees, and make possible to determine what factors play a major role in uprooting the tree. The authors provide recommendations on what base data must be obtained for tree stability tests, and what safety factor should be chosen for the calculations.

By means of the method of calculation can be solved in cases where the tree is no longer available, but there are pictures of it. So the authors make an attempt to describe some interesting instances of tree stability, e. g. paintings of Ivan Shishkin, or other painter, or photos of trees of remote landscape. The method can be used for engineering tasks or for forensic problems too.

Key words: solitary tree, urban trees, wind throw, tree stability

INTRODUCTION

The large size of trees bring about increasing disagreeable effects. The falling branches and first of all the fallen trees can cause a hazard to human life and property. These trees should be intensively operated and cared for, but the difficulty is that the properties of old trees, especially the development of biomechanical stability is not well-understood (Wessolly & Erb, 1998, Coder, 2010).

The technological progress of the last years helped the development of devices for testing mechanical safety and not visible internal defects of trees. Several of these instruments are now available to urban foresters and arborists. Nevertheless in certain cases can not be used these equipments. Such is the case when the instruments is not available, or is not enough space to use its. Often happens the ones that do not have the tree, because it had fallen formerly. These cases occur in forensic assessment reports.

We were looking for easily measured and calculated method, so that the possibility of the tree falling to know.

MATERIAL AND METHODS

The study of the solitary trees in urban areas took place between 2012-2014.

The selection of trees based partly on judicial cases and in part because some of the trees appeared to be in risky locations (Fig. 1.). The trees are located in Western Hungary and in Budapest, and its surrounding area, on streets, gardens and parks.

The static tests occured by 65 individual trees of 25 species with a detailed measurement of 23 structurally important features (e.g. height, dbh, crown diameter, crown height), and the assessment of forces acting on each tree. In addition, 5 variables for each tree came from databases (such as density of wood), and 10 variables were the results of calculations. These variables together include the scope of the data required for the analysis of tree resistance against force of the wind. The attached table (Table 1.) shows the basic data.

The effective root zone was measurable with tape exactly for eight trees that were uprooted by wind (Fig. 2.), so these trees were used to demonstrate a cost-saving tree test procedure developed by the authors. Tree resistance was calculated in Excel using input values from data that were either directly measured or obtained from databases as the below detailed method. The wind load was calculate with the international standards EN 1991-1-4 (2005). The principle of the calculation is the balance of forces having an effect on the tree (Fig. 1.). The wind force induces the falling force, and the root system exerts the resistive force.

When the bending moment of the wind force is equal to the bending moment of the resistive force is the tree stable, that is, the safety factor is 100%. Because the measured data are inaccurate, the safety factor should be chosen to 150%. The function of the bending moment on the balance was already known, but the measurement of the factors haven't standardized. The formulas of the tree stability method can be seen in Table 1.

Table 1. (*in parts the more important parameters*)

	dimen-sion	Kőszeg Square Jurisich	Zrenjanin Gradška Basta	Shishkin, Kama river	Shishkin, Old oak
data / *species*		*Tilia cordata Mill.*	*Picea engelmannii Parry*	*Pinus sylvestris L.*	*Quercus robur L.*
Height (h)	m	20	17	23	12
DBH ($D_{1.3}$)	cm	64	26	38	70
Volume of tree (V_t)	m^3	3.31	0.52	1.33	3.7
Crown drag coefficient (C_c)	-	0.35	0.45	0.25	0.25
Stem drag coefficient (C_s)	-	0.7	0.7	0.7	0.7
Critical wind speed *(by 150% safety f)*	m/s	18		32	38
Gust velocity at the location (v_m)	m/s	14.9	14	17.5	18
Anchoring depth (roots) (n)	m	0.4	0.7	0.5	1.1
Distance of axis of rotation from stem (a)	m	1.8	1	2.2	3.7
Inclination of stem (α)	°	90	75	75	90
Crown area (A_c)	m^2	114	25	20	60
Gravity force of tree $F_t = V_t * \rho_t * g + F_f$	N	16 486	3.25	6 924	36 517
Velocity pressure / turbulence (q_p) (Eurocode, 2005)	N/m^2	463	175	616	350
Wind force on crown $F_c = C_c * A_c * q_p$	N	18 454	1 969	3 082	5 223
Weight force of tree bending moment (M_t)	Nm	29 674	3 260	11 581	135 113
Bending moment of wind $M=F_c*(l+n)+F_t*(l+n)/(2*tg\alpha)$	Nm	136 558	30 808	106 144	44 917
Volume of root system (V_r)	m^3	3.75	2.2	7.6	47.31
Weight force of root system (F_r) $F_r = V_r * \rho_s * g$	N	44 163	29 124	89 498	533 719
Bending moment of root system ($M_r=F_r*a$)	Nm	79 493	29 124	196 897	1 974 761
Bending moment of tree M_r+M_t	Nm	109 167	32 384	208 478	2 109 874
Stability	%	79.9	105.1	196.4	721

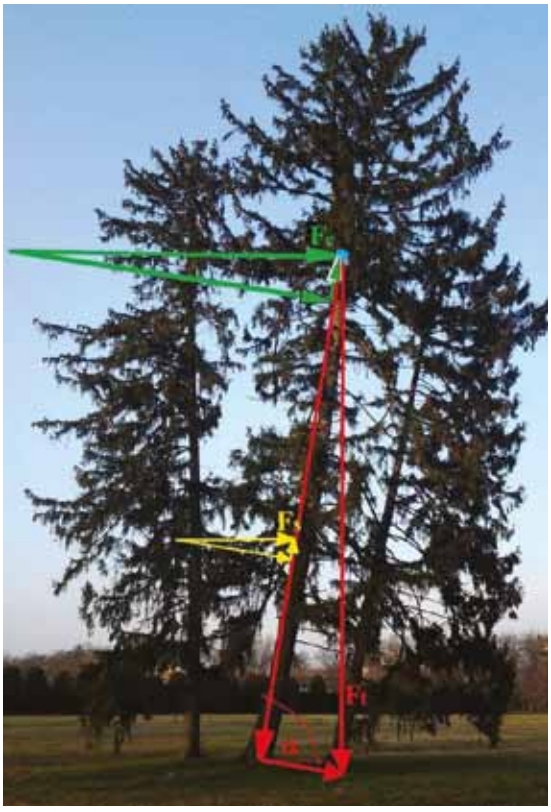

Fig. 1. Norway spruce (*Picea abies* Karst.) by Palace Esterházy Fertőd. On the picture can be examined the components of the wind force. (Source: Authors)

With this method can be measured data not only at the site, but also from photographs (Fig. 3.-4.-5.) moreover from pictures (Fig.6.-7.).

About photos

The authors had the opportunity to examine a photograph from Zrenjanin, about a fallen tree beside Gradska Bašta. The photos were found in internet (www.glassrbije.org, Google Earth and www.rtv.rs/sr_lat/vojvodina/zrenjanin).

Fig. 2. Fallen Small-leaved Lime (*Tilia cordata* Mill.) on the Square Jurisich, Kőszeg. (Source: Authors)

Fig. 3. On the left side of photo stays the Engelmann spruce. It can be seen the tree is a little inclined. The photo was taken in 10/01/2012. (Source: www.glassrbije.org)

Fig. 4. A photo from City centre of Zrenjanin, with the spruce. The photo was taken in 6/22/2013. (Source: Google Earth)

Fig. 5. The fallen spruce. The photo was taken in 14/05/2014. (Source: http://www.rtv.rs/sr_lat/vojvodina/zrenjanin/zrenjanin-orkanski-vetar-rusi-drvece_486181.html

About the photos (Fig. 3.-4.-5.) the following can be assessed:

The tree was Engelmann spruce (*Picea engelmannii* Parry) (Fig. 3.), a little inclined by the dominant wind (Fig. 4.). The from photo measurable data are in the Table 1.

About paintings

On the Fig. 6.-7. can be examined in former times lived trees.

On the painting (Fig 6.) is presented a slender Scotch pine with artistic tools, but realistic. The tree has unusual short crown, and stands in solitaire (free) position.

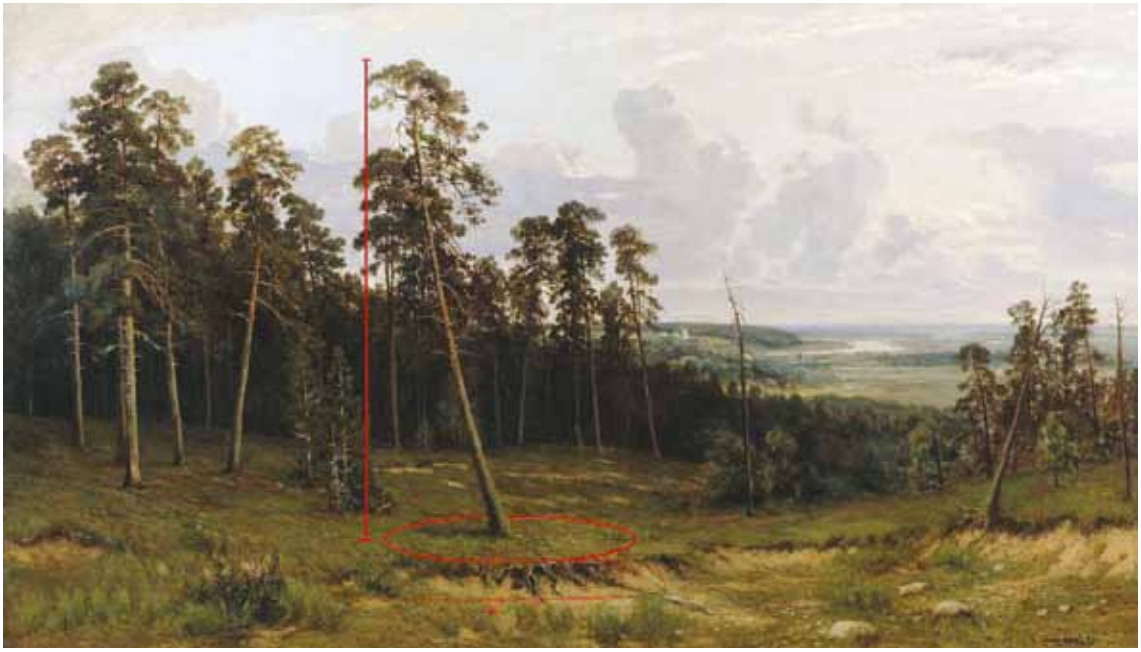

Fig. 6. Shishkin: Pine forest by River Kama, 1877. Богатый лог (Пихтовый лес на реке Каме). (Source: Иван Иванович Шишкин. Издательство Академии Художеств СССР. 1964 Москва)

The precise representation enables the tree to perform the test measurements and calculations. So that in the painting can be assessed the required dimensions, and the extent of the effective root zone, because the artist presented the root system by using a landslip.

On the Fig. 7. is painted an old oak, whose crown is broken, and the root system is exposed by erosion. The effective root system is highly visible. The tree height, DBH and the parameters of the crown of both trees descriptive geometry editing has been determined (Table 1.).

RESULTS

From the measured data of some falling trees, like the its of the Lime in Kőszeg Square Jurisich could be established the equations of the tree stability.

With this method could be determined, what data should be collected about the fallen tree. Than these data can be measured by photogrammetrical methods from precise photos and pictures. So that could be calculated the stability of the formerly falled tree, e.g the Engelmann spruce in Zrenjanin, and due to the landslipe or erosion visible root system of Scotch pine and Pedunculate oak, which was realistic painted by Shishkin.

The stability of the Scotch pine and the oak was high, but the Lime and the Spruce was low (See the Table 1.).

Fig. 7. Shishkin: Old oak. 1866. Старый дуб. (Source: Иван Иванович Шишкин. Издательство Академии Художеств СССР. 1964 Москва)

DISCUSSION

Various indicators and ratios derived from measured and calculated data for a given tree can help assess its health and static loads.

So the Lime in Kőszeg (Fig. 2.) had too shallow root system, like a plate-root systems. The genius *Tilia* has in general heart-root system. Its case was an unknown baroque channel under the tree, and the removed cobblestones due to building operation. So that has reduced the depth of the root system hereby the counterweight against the wind force.

The Engelmann spruce in Zrenjanin Gradska Bašta (Fig. 3., 5.) was inclined, so the centre of gravity was eccentric and near a building developed the tree a limited root system, whose result the safety factor got low. In the time of the wind throw was in the country a stormy weather caused corner eddies (Gromke & Ruck, 2007), that occasioned a wind throw.

Although the Scotch pine on the Fig. 6. is slim and has high center of gravity and the root system locates partly on the surface, it proved to be stable. This is due to the extremely small crown surface.

The roots of the oak are the Fig. 7. also partly free, but the center of gravity is very low, and the root system is very wide, so the lifting arm of counter-force is enormous.

In addition to the basic tree data, this new method requires careful determination of two very important pieces of information: **wind speed** and the extent of **the effective root system**. The process is partly similar to other tree resistance calculations in accounting for static loads and forces of resistance and comparing the bending moment to the resistive moment of the tree. The difference is in the interpretation of the individual factors and their calculation methods (e.g., EUROCODE wind load calculation, determination of crown surface, modified aerodynamic factors, components of forces and the extent of the root zone). This process is particularly suitable for identifying the causes and course of the catastrophic failure of a tree even if the subject tree is no longer available.

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HARMFUL ORGANISMS IN AGRICULTURE

ОРГАНИЗМЫ-ВРЕДИТЕЛИ
Б СЕЛЬСКОМ ХОЗЯЙСТВЕ

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SSR MARKER ANALYSIS INDICATES THE ORIGIN OF *Monilinia fructicola* ISOLATES IN SERBIA?

Jovana Hrustić[1*], Milica Mihajlović[1], Aleksandra Bulajić[2], Branka Krstić[2], Goran Delibašić[2], Andrea Patocchi[3], Maya Jansch[3] and Brankica Tanović[1]

[1]Institute of Pesticides and Environmental Protection, Belgrade, Serbia
[2]Faculty of Agriculture, University of Belgrade, Belgrade, Serbia
[3]Agroscope Changins-Wadenswil (ACW) Research Station, Wadenswil, Switzerland
**jovana.hrustic@pesting.org.rs*

ABSTRACT

Monilinia fructicola is considered to be the most destructive pathogen of the genus *Monilinia*. It is widely spread pathogen of stone fruit in North America and Australia. In European countries, *M. fructicola* is on the A2 EPPO List of pests that are locally present in the EPPO region and recommended for regulation as quarantine pests. Regardless the patogen's status, many major stone fruit growing countries in Europe reported its presence recently. In Serbia, the pathogen was firstly detected in 2011 on detached fruits, but its presence in the field was not proven. Further investigation in the period 2012-2014 confirmed presence and spreading of *M. fructicola* in Serbian orchards.

Simple sequence repeat (SSR) marker analysis is commonly used to assess genetic diversity in a given population. For studing the diversity of *M. fructicola* population, five SSR markers (CHML5, CHMFc1, CHFc4, CHMFc5, and CHMFc12) were developed recently. In the present study, these markers were used to analyze haplotypes of three isolates of *M. fructicola* from Serbia from two different locations and to compare them to the haplotypes originating from Switzerland, Spain, Italy, France, and the USA. All five SSRs were multiplexed in a single PCR reaction. The marker CHFc4 revealed two alleles (216 and 223 bp in length), while the other markers detected only one allele, different in lenth: CHML5 (220 bp), CHMFc1 (173 bp), CHMFc5 (86 bp) and CHMFc12 (160 bp), respectively. These results showed that Serbian isolates belonged to two different haplotypes. The same haplotypes were previously detected in Italy, suggesting possible pathway of the introduction of this species to Serbia.

Key words: quarantine pest, introduction, genetic diversity

INTRODUCTION

Monilinia spp. are well-known plant pathogens that endanger pome and stone fruit production worldwide. They are among economically the most important limiting factors for fruit production all over the world. Under favourable weather conditions, when rainy period coincidence with blooming and fruit ripening, consequence of brown rot incidence in stone fruit production might be very severe. However, the most severe damage and yield losses occure due to either pre- and post-harvest fruit rots (Hong et al., 1997).

Three species of *Monilinia* spp. are considered to be economically significant: *M. fructigena* (Aderhold and Ruhland) Honey, *M. laxa* (Aderhold and Ruhland) Honey and *M. fructicola* (Winter) Honey. Among them, *M. fructicola* is the most destructive species causing blossom and twig blight, as well as

fruit rot (Fulton et al., 1999). It is officially on A2 EPPO List of quarantine pest organisms in Europe (http://www.eppo.org/QUARANTINE /quarantine.htm) and on 1A part I list of quarantine pest organisms in Serbia. However, over the last 13 years this species has been detected in several European countries as well as in Serbia. Thus, recent reports of this pathogen came from France (EPPO, 2002), Hungary (Petroczy and Palkovics, 2006), the Czech Republic (Duchoslavová et al., 2007), Italy (Pellegrino et al., 2009), Spain (De Cal et al., 2009), Switzerland (Bosshard et al., 2006; Hilber-Bodmer et al., 2010), Slovenia (Munda and Viršček Marn, 2010), Slovakia (Ondejkova et al., 2010), Germany (EPPO, 2010), Poland (Poniatowska et al., 2013), Serbia (Vasić et al., 2012; Hrustić et al., 2013) and Croatia (Ivić et al., 2014). Despite its wide distribution, *M. fructicola* is still a quarantine plant pathogen in Europe.

Due to the fact that *M. fructicola* was recently introduced in Europe, genetic diversity within and among populations of *Monilinia* species has been widely studied. A large number of different molecular techniques, from inter-simple sequence repeat (ISSR), restriction fragment lenght polymorphism (RFLP), amplified fragment lenght polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) fingerprinting to simple sequence repeat (SSR) marker analysis, revealed differences among *Monilinia* species. Within the genus *Monilinia*, genetic diversity was the most frequently investigated in *M. fructicola* (Fulton et al., 1999; Fan et al., 2010; Gril et al., 2010; Villarino et al., 2012), mostly because of the ability of this species to produce ascospores from pseudosclerotial mummified fruit (Byrde and Willetts, 1977).

SSR marker analyses is commonly used to assess genetic diversity in a given population and can be very useful to identify ways by which the pathogen spreads or being spread. On the basis of SSR analysis Jansch et al. (2012) showed that in *M. fructicola* some European countries originated from at least two independent introduction from the USA.

Until recently, the brown rot in Serbia was caused by two species. *M. laxa* and *M. fructigena,* that were widespread and appeared every year. In 2011, a third species, *M. fructicola*, was detected in nectarine fruit originating from green markets selling local fruit products. Taking this into account recent discovery of *M. fructicola* in Serbia, the aim of this study was to determine whether SSR marker analysis could indicate the origin of *M. fructicola* isolates in Serbia as well as whether trade could be responsible for introduce of *M. fructicola* into Serbia.

MATERIAL AND METHODS

Isolates

Three *M. fructicola* isolates (NPGM, NPUD1 and NPUD 2), selected from 249 *Monilinia* spp. isolates, derived from mummified fruit, infected twigs, and rotted fruit of stone fruit in Serbia and identified based on pathogenic, morphological and molecular characteristics in our previous investigation (Hrustić et al., 2015) were used in this study.

SSR marker analysis

Jansch et al. (2012) developed SSR markers for genetic diversity assessment of *M. laxa* and *M. fructicola* populations using the protocol of Brunner and Frey (2004). Five SSR markers (CHML5, CHMFc5, CHNFc1, CHMFc4 and CHMFc12) were multiplex using the Multiplex PCR kit. The total PCR reaction mix (final volume 10 µl) consisted of 2 µl DNA, 5 µl Multiplex PCR Master Mix, 1 µl of Q-Solution, 1 µl sterile water and 1 µl of the primer mix (final concentration: CHML5f/r 1,875 µM, CHMFc1f/r 5 µM, CHMFc4f /r 1,25 µM, CHMFc5f/r 1,875 µM i CHMFc12f/r 3,75 µM). Amplification were performed in a termocycler (SensoQuest) with an initial denaturation at 95°C for 15 min followed by 35 cycles of 94°C for 40 s, 50°C for 90 s, 72°C for 90 s; ending with 30 min at 60°C and a final hold at 10°C. The ABI PRISM®3100 DNA capillary sequencer (Applied Biosystems) was used for fragment analysis. A total of 0.8 µl of a 1:20 diluted PCR product was transferred to 15 µl of formamide containing 0.25 µl of the fluorescent GeneScan™-500-LIZ™ size standard (Applied Biosystems). The reactions were then denatured for 5 min at 95°C, rapidly cooled in the freezer, and loaded on the sequencer. For data analysis GeneMapper™ v. 4.0 (Applied Biosystems) was used.

In order to identify haplotypes of *M. fructicola* isolates originating from Serbia, the obtained allele patterns were compared to those described by Jansch et al. (2012).

RESULTS AND DISCUSSION

Variability of SSRs producing amplicons from three *M. fructicola* isolates originating from Serbia was evaluated by using five markers in a single PCR reaction. SSR markers CHMFc1, CHMFc5, CHMFc12 and CHML5 revealed only one allele each (173, 86, 160 and 220 bp, respectively) in the investigated isolates of *M. fructicola*. On the other hand, marker CHMFc4 detected two alleles (216 and 223 bp) (Table 1). Therefore, the isolates of *M. fructicola* from Serbia belonged to two different haplotypes.

Table 1. Allele (in bp) composition of *Monilinia fructicola* haplotypes found in Serbian samples

Isolates	CHMFc1	CHMFc4	CHMFc5	CHMFc12	CHML5	Haplotypes according Jansch et al. (2012)
NPUD1	173 bp	216 bp	86 bp	160 bp	220 bp	US50
NPUD2	173 bp	216 bp	86 bp	160 bp	220 bp	US50
NPGM	173 bp	223 bp	86 bp	160 bp	220 bp	ITA01

According to Jansch et al. (2012) appointment, studied isolates NPUD1 and NPUD2 originating from the same location have the same haplotype, identified as US50. The third isolate (NPGM) originating from different location was ITA01 haplotype (Table 1). The same haplotypes were previously found; US50 in the USA and Italy and ITA01 in Italy only (Jansch et al., 2012). This finding suggests two independent entries of *M. fructicola* into Serbia.

This study represents the first investigation of *M. fructicola* isolates from Serbia using SSR marker analysis as a powerfull tool for genetic diversity studies. SSR markers have successfully been used for the characterization of genetic diversity of many plant pathogenic fungi including *Verticillium dahliae* (Berbegal et al., 2011), *Ascochyta rabiei, Ceratocystis fimbriata, Macrophomina phaseolina, Puccinia graminis* and *P. triticina, Sclerotinia subarctica* and *S. sclerotiorum, Phytophthora ramorum* (Capote et al., 2012).

Intensive studies of *Monilinia* spp. on stone fruits in Serbia started in 2010 (Hrustić et al., 2013). During 3-year period (2010-2012), more than 250 samples were collected from different parts of Serbia and 249 *Monilinia* spp. isolates were derived. The resultes showed that *M. laxa* was by far the most predominant species associated with brown rot, mummified fruit, infected twigs, and fruit rot disease. In 2011 the presence of *M. fructicola* on stone fruits in Serbia was detected for the first time. The isolate was derived from diseased nectarine fruit originating from local green market (Hrustić et al., 2013). In 2012 additional two isolates of *M. fructicola* were found. They were also isolated from symptomatic nectarine fruits from green market. It should be emphasized that during that period, the presence of *M. fructicola* in orchards was not confirmed in spite of intensive survay (Hrustić et al., 2014). Further investigation in 2013 confirmed occurrence of this species in nectarine orchards in Serbia (Hrustić et al., 2015).

Despite the fact that *M. fructicola* is a quarantine pest in EPPO region, its presence has been confirmed during the last 13 years in many European countries (EPPO, 2002; Bosshard et al., 2006; Petroczy and Palkovics, 2006; Duchoslavová et al., 2007; De Cal et al., 2009; Pellegrino et al., 2009; Hilber-Bodmer et al., 2010; EPPO, 2010; Munda and Viršček Marn, 2010; Ondejkova et al., 2010; Vasić et al., 2012; Hrustić et al., 2013; Poniatowska et al., 2013; Ivić et al., 2014). As potential sources of introduction of *M. fructicola* to European countries imports of diseased stone fruit vegetative material for establishment of new orchards and imports of diseased stone fruit during winter for consummation were mentioned (Bosshard et al., 2006; Jansch et al., 2012; Papavasileiou et al., 2014). The import of *M. fructicola* from France and California to Switzerland, and from Spain and Italy to Greece were previously documented (Bosshard et al., 2006; Jansch et al., 2012; Papavasileiou et al., 2014). In addition, the results of surveys indicated that trade might be responsible for introduce *M. fructicola* into Switzerland and Hungary (Bosshard et al., 2006; Petrozcy and Palkovics, 2006). It could be hypothesized that the presence of the pathogen in Serbia could be attributed to the import of fruit or planting material from other countries.

The presence of *M. fructicola* in stone fruit in Serbia represent an important threat to the stone fruit production of the country. Aplication of SSR markers indicated that better quarantine control is needed in order to prevent further enteries. We assume that introduce of new strains of *M. fructicola* would enlardge its genetic variability leading to better adaptation capability and easier spread of the pathogen. In addition, this species grows faster, sporulates more abundantly and has better dispersal ability than the other *Monilinia* species (Villarino et al., 2013). It is also more prone to fungicide resistance development (Yoshimura et al., 2004; Luo et al., 2008; Chen et al., 2013a, b). Therefore, for successful control of the disease, continuous monitoring of the changes in the population structure of brown rot causal agents is required.

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THE INCIDENCE OF VIRUSES IN SERBIAN POTATO SEED PRODUCTION

Mira Starović, Anja Milosavljević, Erika Pfaf-Dolovac, Goran Aleksić, Nenad Dolovac and Slobodan Kuzmanović

Institute for Plant Protection and Environment, Teodora Drajzera 9, Belgrade
E-mail: miragavranstarovic@gmail.com

ABSTRACT

Potato (*Solanum tuberosum* subsp. *tuberosum*) is very important field crop grown in Serbia. A significant number of pathogens, including viruses, can naturally infect potato crops. Virus infections have been associated with potato degeneration and declining productivity of seed stock in the major potato producing countries. Domestic production of potatoes is mainly based on seeds of the class "certified" imported from northern European countries. In this study we examined the annual increase in viral infection in domestic production of seed potatoes in two consecutive years.

A survey to determine the incidence of potato virus diseases was carried out in four major potato growing areas in Serbia. Tubers were harvested from 591 field crops in 25 locations during September 2012 and from 507 field crops in 23 locations during August 2013. Disease incidence was confirmed by the laboratory testing using double antibody sandwich (DAS)-ELISA test on *Potato virus Y* (PVY), *Potato virus A* (PVA) (monoclonal antibody), *Potato leaf roll virus* (PLRV), *Potato virus S* (PVS), *Potato virus X* (PVX) and *Potato virus M* (PVM) (polyclonal antibody) of Bioreba production.

Infection were detected in 477 out of 591 (80.71%) and in 312 out of 507 (61.54%) crops in 2012 and 2013, respectively. In 2012, out of 14775 samples tested by ELISA, 5819 samples were infected by PVY, 184 by PLRV and only one by PVS. Mixed infection PLRV+PVY was detected in 149 samples (1,01%). In 2013, 2654 samples were infected by PVY from 12675 tested samples, and 95 by PLRV. Mixed infection with PLRV+PVY was present in 37 samples (0.25%). Results of laboratory testing showed the occurrence of at least two viruses, with PVY being the most widely distributed, 39.62% and 21.39% of tested samples in 2012 and 2013 respectively. Other viruses identified were less present. In 2012, PLRV was detected in 1.24%. In 2013, 0.75% samples were infected with PLRV, while PVS was detected only sporadically. PVA, PVX and PVM were not detected in any of the samples tested in the years of investigation.

PVY was the most frequently detected virus, detected in 39% and 21% of samples collected in 2012 and 2013, respectively. PVY proved to be endemic throughout Serbia.

Key words: ELISA, seed potato, PVY, PLRV, virus incidence

INTRODUCTION

Potato (*Solanum tuberosum* subsp. *tuberosum*) is one the fourth importance crop in Serbia, with a production in 2012 just over 0,5 million metric tons (Faostat, 2012). Potato are propagated vegetatively from seed tubers. In this way a high number of pathogens can be carried over from one year to the next. Among them 37 viruses appear on potato can play a significant role (Valkonen, 2007).

Domestic production of ware potatoes is mainly based on seeds of the class "certified" imported from northern

European countries. Some significant among of seeds are also produced locally and certified in Serbia after propagation of imported seeds. According to national legislation the tolerance for virus infection is 1% for category "elita" ("E"), 6% for "A" and 10% for "B" category.

The severe crop losses in yield and quality caused by infections originating from infected seed tubers (secondary infections) (Milošević, 2009).

This study was conducted for assessing the relative incidence of PVY and PLRV in a 591 and 507 potato fields in 2012 and 2013 respectively.

MATERIAL AND METHODS

The samples of the potato seed production from 4 districts: Raski, Moravicki, Zlatiborski and Moravicki, were submitted for analysis. Per 100 tubers (represent one semple) were harvested from 591 field crops in 25 locations during September 2012 and from 507 field crops in 23 locations during August 2013.

Tubers were planted in pot and kept for at 25±2°C in the glasshouse condition. Four weeks after plantation a young fully expanded leaves of each shoot of the plantlets were sampling for analysis. Disease incidence was confirmed, by the laboratory testing using double antibody sandwich (DAS)-ELISA test on PVY, PVA (monoclonal antibody), PLRV, PVS, PVX and PVM (polyclonal antibody) of Bioreba production.

The results are presented as the total number of viruses infected plants by districts over the total number of samples with infection below 1% and over 10%, and the percentage of samples in the categories E, A and B in relation to the total number of samples during 2012 and 2013.

Results are presented as the total number of infected plants with tested viruses, single and mixed infection, then the total number of samples with infection below 1% - (elita) and over 10% (can use as seed), and the percentage of categories of produced seed potato: E, A, B and no seed potato (>10%) in relation to the total number of samples per localities during 2012 and 2013.

RESULTS

Of a total of 14775 tested potato plants during 2012, 39.38% were infected by PVY, 1.25% by PLRV AND 1% with both viruses. In 2013 in 20.94% tested plants PVY were proved, in 0.75% PLRV and in 0.29% mix infection (Tab.1).

Results of showed the occurrence of at least two viruses, with PVY being the most widely distributed, 39.62% and 21.39% of tested samples in 2012 and 2013 respectively. In 2012, PLRV was detected in 1.24%. In 2013, 0.75% samples were infected with PLRV, while PVS was detected only sporadically. PVA, PVX and PVM were not detected in any of the samples tested in the years of investigation.

The samples with infection below 1% belong to the category ELITA. The lagers number of samples in the category elita was produced in Zlatiborski district in booth of tested years (2012 - 25.32%, 2013 - 44.76%,), and in Raski district in 2013-40.0%, while in Macvanski district in 2012 wasn't produced any.

The samples with infection over 10% cannot be used as seed. In 2012 in Moravicki district nearly half samples (49.38%) was infected over 10% and in Macvanski 44,44%, while in 2013 in mention districts was about a third of (Tab. 2).

Tab. 1. Survey of potato viruses in Serbia according DAS-ELISA test in 2012 and 2013

Viruses tested	2012		2013	
	Single infection	Mixed infection	Single infection	Mixed infection
PVY	5819	/	2654	/
PLRV	184	/	95	/
PVS	0	/	0	/
PVA	0	/	0	/
PVS	1	/	0	/
PVX	0	/	0	/
PVM	0	/	0	/
PVY+PLRV	/	149	/	37
Uninfected	8772		9926	

Tab. 2. The number of potato seed samples with virus infection below 1% and over 10% of investigations Serbian districts in 2012 and 2013.

		Districts	No. tested samples	No of infected samples over 10%	No of infected samples up to 1%
Year	2012	Moravicki	243	120	45
		Zlatiborski	233	75	59
		Raski	88	32	11
		Macvanski	27	12	0
	2013	Moravicki	176	52	59
		Zlatiborski	210	46	94
		Raski	95	20	38
		Macvanski	24	8	1

[a] Monoclonal antibody detects both the common (PVY^O) and the potato necrotic (PVY^N) strains.

The relative frequencies of produced potato seeds categories in relation to the total number of samples per localities during 2012 and 2013 are shown in Graf. 1.

DISCUSSION

The results obtained through laboratory testing indicated that these viruses is the limiting factor to potato seed production in our country. The presence and the rate of infection with the PVY has been studied over the three decades and its distribution has been epidemic in Serbia and in the wider region (Milošević, 2013). This virus significantly reduce the yield of potato tubers and is the most important limiting factor in the production of seeds potato in Serbia and neighbouring countries (Milošević, 2009), ours investigation proved this fact.

Results of our investigation showed the occurrence that PVY was the mostly severity distributed in 2012 and 2013, then PLRV, while PVS was detected sporadically. According to the previous investigation in Serbia distribution and the intensity of infection of healthy potato plants in Serbia, the most important virus is a PVY, followed by PLRV and S virus PVS (Milošević, 1992; Gavran, 1996; Milošević *et al.,* 2000; 2008; 2012).

The extensive spread of PVY is already became a great threat for the Serbian potato industry. Unfortunately, this occurence is present in some seed potato producing district, such as Moravicki and Macvanski, resulting in the rejection most of the produced potato seed lots which are infected with PVY a high percentage (>10), especially in 2012. Incidence of produced elita category in 2013 was much higher compared to 2012. This can be explained by the fact that the tuber harvest in 2013 was one month earlier than the year 2012. It is necessary to do a more detailed analysis of monitoring the flight of aphids, the impact of altitude and potato cultivars to PVY infection percentage. This analysis would significant contributed to recommendations for successful seed production, such as Racliffe and Radsdale (2002), already reported. According to their results vector population dynamic in relation to the presence of virus sources, host plant availability and resistance will determine the onset, progress and the final incidence of the disease.

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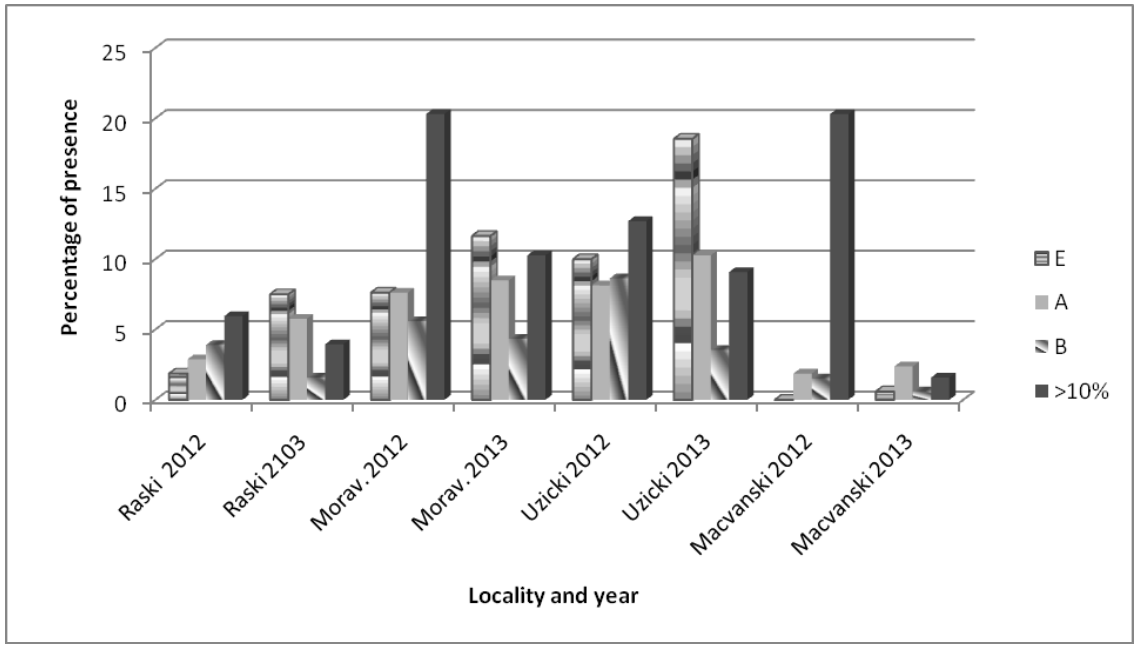

Graf. 1. Incidence of different category of produced seed potato in relation to the total number of samples per district during 2012 and 2013.

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PRECISION AGRICULTURE IN POLISH INTEGRATED PLANT PROTECTION

Danuta Sosnowska and Żaneta Fiedler

Institute of Plant Protection – National Research Institute
W. Wegorka 20 str., 60-318 Poznan, Poland
e-mail: Danuta.Sosnowska@iorpib.poznan.pl
Z.Fiedler@iorpib.poznan.pl

INTRODUCTION

Polish accession in the European Union has introduced a number of legislative acts in the force. One of them is Directive 2009/128/EC of the European Parlament and the Council of 20 October 2009 establishing a framework for the Community action to achieve the sustainable use of pesticides. Article 14 this Directive says that member states shall take all necessary measures to promote low pesticide input pest management, giving wherever possible priority to non-chemical methods. From January 1st 2014 professional users of pesticides should switch to practices and products with the lowest risk to human health and the environment. Farmers have to use integrated pest control only.

Precision agriculture is a method of intensification in agricultural production with respect to the principles of sustainable development. Plant protection with use of the instruments of precision agricultures is the element of agricultural production in which instruments can be easily balanced by the obtained benefits.

Idea of the integrated pest control fits to the system of precision agriculture.

In this paper, one of the elements of agriculture production such as integrated plant protection will be discussed.

INTEGRATED PLANT PROTECTION

Integrated Plant Protection (IPP) is part of Integrated Pest Management (IPM).

IPM is an ecosystem – based strategy that focused on long – term prevention of pests or their damage through a combination of methods such as biological control, habitat manipulation, modification of cultural practices, and use of resistant varieties. Pesticides are used only after monitoring indicates they are needed according to established guidelines, and treatments are made with the goal of removing only the target organism. Pest control materials are selected and applied in manners that minimize risks to human health, beneficial and non target organisms, and the environment.

General principles of IPM are:

a) The prevention and/or suppression of harmful organisms should be achieved or supported among other options especially by:

- crop rotation
- use of adequate cultivation techniques
- use of resistant/tolerant cultivar and standard/certified seed and planting material
- use of balanced fertilization, liming and irrigation/drainage practices
- perverting the spreading of harmful organisms by hygiene measures
- protection and enhancement of important beneficial organisms.

b) Harmful organisms must be monitored by adequate methods and tools, where available.

c) Based on the results of the monitoring the professional user has to decide whether and when to apply plant protection measures.

d) Sustainable biological, physical and other non – chemical methods must be preferred to chemical methods if they provide satisfactory pest control.

e) The pesticides applied shall be as specific as possible for the target organism.

f) The professional user should keep the use of pesticides and other forms of intervention to levels that are necessary.

g) The use of multiple pesticides with different modes of action where the risk of resistance of pests to chemical pesticides in known.

Idea of the integrated pest control fits into system of precision agriculture, because help farmers make the best decision with regard to planting, fertilizing, pest control and harvesting crops. In plant protection crop management is still in the experimental phase.

PEST CONTROL IN PRECISION AGRICULTURE

In IPP, monitoring and correct pest identification helps farmers to decide whether management is needed. Monitoring means checking farmer field, landscape, forest to identify which pests are present, how many there are, or what damage they've caused. Correctly identifying the pest is key to knowing whether a pest is likely to became a problem and determine the best management strategy. After monitoring and considering information about the pest, its biology, and environmental factors, we can decide whether the pest can be tolerated or whether it is a problem that warrants control. If control is needed, this information also helps to select the most effective management methods and the best time to use them.

Insect trapping is another good tools to assist a grower in monitoring the insect pressure within a given field or crop. For the accurate determination of the pest the Institute of Plant Protection - National Research Institute (IPP-NRI) in Poznan apply different tools: yellow traps, light trap, Johnson's aspirators, pheromone traps, sweet nets.

Important is also the visual method, which provides the concrete data about the abundance of insect species initial population or the intensification of its occurrence during the crops vegetative season. A direct observation might be soil analysis for the presence of beetle larvae, pupae of phytophagous insects, inspection of plants for beetles (*Coleoptera*) or their development stages, as well as to detect a possible invasion of winged forms, for example, aphids (*Aphididae*) onto the cultivated plants.

Insect catching in used for diagnostic purposes, to determine which species occur on a particular area, or a particular plant damaging it. The method is also used for flight dynamics control and determining the gradation stage of a species. Phytophagous insects are caught using different methods and various kinds of equipment:

1. light traps, which are used mainly to catch the nocturnal butterflies (*Lepidoptera*, *Noctuidae*). They have a glow discharge lamp (250 W), powered by alternating current, that serves as decoy. It allows efficient catching of nocturnal imagines of the *Noctuidae* moths.

2. yellow water – pan traps. These traps (Moricke's traps) are based on the presumption that some species e.g. beetles (*Coleoptera*) are attracted to the yellow color inside the pan. It is the best way to monitor invasions and activity of beetles.

3. yellow sticky traps

Insects are attracted by a yellow decoy and trapped by glue distributed on its surface. These traps are to monitor flights and abundance of *Diptera* flies.

4. Barber's trap

Is a plastic container consisting of a plastic cup, a funnel and a jar. It is placed in the field ground. These traps are used while observing pests crawling on the ground surface, such as *Coleoptera – Curculionidae* and others.

5. food traps, are used when the soil is analyzed for the presence of *Elateridae* larvae. Food traps are usually plastic containers with multiple holes and a cover, filled with food, e.g. potato tuber, carrot.

6. collecting nets are used for catching butterflies sitting on the plant flowers.

7. pheromone traps are based on the use synthetic pheromone like compounds. Pheromones are hormonal substances emitted by female insect. They attract males only. Therefore, only the insect attracted to a given pheromone will be present in these traps. Pheromones can also be used to confuse insects by making them mates are in the area. This can help to reduce populations of the next generation.

8. Johnson aspirator.

Is used for catching aphids and measuring their migration density. It is of especially great importance for early indications, especially of aphid species able to transfer pathogenic viruses onto various crops.

The precision management of insect pests relies on the same three elements (information, technology and management) that are important in the precision management of other crop production variables (such as soil nutrients). Constructing maps of insect pests is much more difficult because insect populations generally

are spatially dynamic (changing density and location over time) and the methods that exist for mapping their distribution tend to be complicated, labor intensive, and uneconomical.

Precision agriculture can help in managing crop production inputs. In an environmentally friendly way. In plant protection pesticides should be applying only where and when they are needed, should reduce environmental loading. In plant protection application of plant protection chemicals cannot be avoided, by they have to be used responsibly; their use has to be economically beneficial and has to take into account the social aspect.

In plant protection important is method of forecasting and signaling for chemical pest control. Important is also decisions regarding time for chemical pest control.

In the Institute of Plant Protection – NRI in Poznan short – term forecasting using the computer program based on a verified mathematical model was development. This method is used to protect cereal crop from leaf beetles (*Oulema* spp.). In many countries, one of the most economically significant pests on cereal crops are leaf beetles (*Oulema* spp.) (Wellso 1985). In Poland, there are two different species of leaf beetle – *Oulema melanopus* and *O. gallaecina* from the *Chrysomelidae* family (Walczak 1990). Each year the beetles and the larvae damage the assimilating surfaces of cereal crops leaves, causing losses in yields (Walczak et al.2009).

Research was conducted in the years 2006-2009. The research consisted in examining the usefulness of development mathematical models in the form of multiple curvilinear regressions. The model has been developed with the aim of being helpful in making decisions regarding time for cereal leaf beetle control. The basis for developing those models was statistical analysis of resulting obtained during a 5-year long period of *Oulema* spp. rearing in a phytotron and 3-year long period of rearing them in natural condition (Walczak 2008).

The model takes into account the influence of air temperature and humidity on the length of the incubation period of cereal leaf beetle. In forecast the length of the egg incubation period for example the length of the period of mass egg laying and mass larvae hatching is important. This mathematical model is very useful for the farmers or their advisors when determining the data of the treatment. In saves time and eliminates a mistake often made by producers, who decide to start leaf beetle control once considerable parts of the leaves surface have already been damaged. Treatments not conducted at optimum times are simply not profitable.

DISEASE CONTROL IN PRECISION AGRICULTURE

The identification and quantification of the dynamics of disease spread have been developed extensively and are assumed to be the result of spore dispersal, production, and the removal of infection sites due to previously infected plant tissue. The potential to predict where the foci are likely to occur be an important tool in the precision application of fungicides, especially protecting fungicides that cannot stop the infection once it has begun.

By predicting the advancing wave of infection, it would be possible to design precision farming fungicide applications that would enhance disease control and reduce the potential of resistant development. For example, the areas with visible and latent infections could be treated with a systematic fungicide, while a different spray could be applied to the invisible latent infections, as well as to adjacent infection sites in front of the infection wave that may have been contaminated with spores but not yet infected. This differential fungicide application would not only reduce the chance of resistance development by the pathogen, but would also reduce application costs, as many of the protectant fungicides are cheaper than the systemic. Recent advances in GPS and application equipment have set the stage for rapid advancement of the application of GPS technology to disease control.

For determination of diseases Institute of Plant Protection – NRI in Poznan use volumetric spore trap. Institute has been providing the pest and disease regional monitoring since 2005. The results are published on the Institutes' website (www.ior.poznan.pl) under "Sygnalizacja Agrofagów" (Pests/diseases signalization). Except information about first appearance and next developmental stages the above website provides information regarding pests and diseases biology too. Such information helps the producers eliminate their individual situations on the field.

WEED CONTROL IN PRECISION AGRICULTURE. DECISION SUPPORT SYSTEM

Controlling weeds is essential in order to obtain optimum yield and quality of cereal plants. Among the issues of precision agriculture the most important ones are Decision Support System (DSS) for prognosis of pest, weed and disease incidences, characterization, detection and identifications systems for precise determination of

the spray target and navigation systems used to control the executive tools and devices for spray application.

Institute of Plant Protection – NRI in Poznan participates in the UE project "Joint use of Danish Decision Support System (DSS) for minimizing use and outflows of herbicides (DSS Herbicide". The aim of the project is to minimize the environmental impact of herbicide treatments in the Baltic Sea Region. The project has a particular focus on herbicides in winter wheat in the Baltic region (northern Germany, northern Poland and southern Denmark).

DSS Herbicide is using a three-step "decision engine":

a) assessment of the need for weed control

b) selection of single herbicides and calculation of dose rates

c) optimization of tank mixtures.

The overall idea of the project is to adapt a web-based Decision Support System (DSS) for farmers that has been developed and successfully applied in Denmark.

The system helps to optimize and reduce the use of herbicides at the farm level. The activities involve in a first step the creation and incorporation of basic data that allow the adoption of the system to winter weed and the agronomical, biological and lingual conditions in Poland and Germany. The final result will be two fully operational, web-based DSS in Poland and Germany, and an extended/improved system in Denmark. They will be promoted to farmers via an extensive promotion campaign, involving continuous feedback processes with farmers/farm advisors and support by professional advisors.

SPRAY APPLICATION EQUIPMENT IN PRECISION AGRICULTURE

In precision agriculture the equipment and techniques are continuing to be developed and improved. The proper choice and use of spray application equipment have direct influence on application efficacy. Several recent developments have been aimed at modifying existing equipment to increase biological performance of pesticides, deposition efficiency of droplets while reducing the potential for drift. In general, this has been obtained by using new nozzles, air-assist system, or some kind of shield to overcome the drift-producing air currents and turbulence that occur around the nozzle during spraying. The goal in the proper application of pesticides is to achieve a uniform spray distribution while retaining the spray droplets within the intended target area. In this way have been created a new generation of nozzles (low drift, venturi- air induction, twinjet and other) which decrease chemical spray drift on the one hand and improve the pesticide efficacy on the other hand.

The new electro-aerosol technology tested in Institute of Plant Protection – NRI in Poznan utilizes liquid in the form of an electrostatic spray loaded with high-speed stream. The large initial velocity stream of aerosol particles provides deep penetration of the canopy, and the introduction of electric charge on the particles leads to an increase in the surface coverage spray target. Developed sprayer is designed primarily for the protection of high and dens plant canopy, e.g., corn, rape-seed oil or potatoes up to the soil surface. Results indicate on the large usefulness of the new application technique (supersonic jets with electrostatics loading) in agricultural practice against control of European corn borer (*Ostrinia nubilalis* Hbn.) on the corn, rape blossom beetle (*Meligethe saeneus*) and rape-seed weevil (*Ceutorhynchus assimilis*) in winter oilseed rape, and spray coverage of potato plants with fungicide during control of late blight (*Phytophthora infestans*). The usage of limited spray volume to approx. 40 l/ha as a very fine spray quality with very large initial energy, and also the possibility of electrostatic loaded of the spray droplets assures the attaching of plant protection product to lower parts of plants canopy also on the bottom side of leaf blade. This new technology gave new possibilities of effective chemical protection of many cultivation crops against agrophages (pest and disease) in dense and high crops.

FUTURE OF PRECISION AGRICULTURE IN IPM IN POLAND

Precision agriculture includes all those agricultural production practices that use information technology either to tailor input use to achieve desired outcomes, or to monitor those outcomes (e.g. variable rate application, yield monitors, remote sensing).

One of the challenges is to show that precision agriculture can have a positive impact on the environment. Precision farming is a technology that will modifies existing techniques and incorporates new ones to produce a new set of tools for management. Maps that characterize the spatial distribution of crop production variables such as soil nutrients, weed populations, and harvest yields will be the most important components in the precision approach to agriculture. Technologies such as Global Positioning System (GPS) enable farmers to develop and use the maps with their – map - sensitive farm equipment so that they can proscriptively apply plant protection and nutrients.

Nanotechnology has developed tremendously in the past decade and was able to create many new materials with a vast range of potential applications. Nanotechnology is defined as the manipulation and control of matter with dimension of 1-100 nanometers (billionths of a meter). At that small scale, particles have different properties. The future of precision agriculture will be nano-pesticides. The application of these products would be the only intentional diffuse input of large quantities of engineered nano-particles into the environment. Nano-pesticides may reduce environment contamination through the reduction in pesticide application rates and reduced losses. The secondary metabolites in plants have been used in the formulation of nanoparticles through increase the effectiveness of therapeutic compounds used to reduce the spread of plant diseases, while minimizing side effects for being: rich source of bioactive chemicals, biodegradable in nature and non-polluting (eco-friendly). Some nanoparticles like silver have promise action against some bacteria in plants (AbdulHammed 2012).

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GRAPEVINE FLAVESCENCE DORÉE PHYTOPLASMA IN SOUTH-EASTERN SLOVENIA AND ITS VEKTOR AMERICAN GRAPEVINE LEAFHOPPER (*Scaphoideus titanus* Ball)

Karmen Rodič[1], Magda Rak Cizej[2], Erika Orešek[3], Domen Bajec[1] and Andreja Peterlin[1]

[1] Agriculture and Forestry Chamber of Slovenia, Agricultural and Forestry Institute Novo mesto, Plant protection service, Šmihelska cesta 14, SI - 8000 Novo mesto, Slovenia
[2] Slovenian Institute of Hop Research and Brewing, Cesta Žalskega tabora 2, SI – 3310 Žalec, Slovenia
[3] Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection, Dunajska cesta 22, SI-1000 Ljubljana, Slovenia
karmen.rodic@gov.si

ABSTRACT

Grapevine flavescence dorée (GFD) is a quarantine grapevine disease caused by phytoplasma. Due to its great impact on the quality and quantity of the crop, GFD is listed in the Annex II.A.II of the Council Directive 2000/29/EC. It is an epidemic disease characterized by its rapid spread within vineyards due to vine-to-vine transmission by the vector american grapevine leafhopper (*Scaphoideus titanus* Ball). Infected grapevines can die within a few years. Beside GFD, in Slovenian vineyards also Bois noir phytoplasma (BN) is present.

In south-eastern Slovenia, *S. titanus* was first recorded in 2005. Since then, it has spread quickly throughout the whole wine-growing region Posavje and is now present in the entire territory of Slovenia. The first finding of GFD in south-eastern Slovenia was confirmed in 2008 (Piroški vrh near Brežice). In 2010, the first outbreak of the disease was discovered in Dolenjska region in the vicinity of Straža near Novo mesto.

Removal of infected vines and treatment of *S. titanus* are the most important phytosanitary measures for prevention of spread of GFD. In accordance with the Rules on the prevention of spread and eradication of Grapevine flavescence dorée and the Official action plan, treatment of the vector is obligatory in all vineyards in the demarcated areas and in all nurseries and mother plantations.

Monitoring of *S. titanus* in the winegrowing region Posavje which is carried out by the plant protection service is crucial for determination of the optimal term of treatment. During the years 2012 - 2014, larvae hatching, their moulting periods and occurrence of adults were monitored as well as the beginning and the top of occurrence of adults by yellow sticky traps. Duration of the length of different developmental stages was investigated also in controlled conditions.

Key words: Quarantine disease, bionomics, spreading, monitoring, growing chamber

INTRODUCTION

Official survey on Grapevine yellows in Slovenia has been carried out since 2002. First finding of Grapevine flavescence dorée phytoplasma (GFD) was confirmed in 2005 near Koper on the variety Pinot gris. In the following years positive grape wine plants were found only in Slovenian Istria, in the year 2008 GFD was found for the first time in South eastern Slovenia. Further confirmations of GFD were connected with the spread of *S. titanus* which is now present everywhere where grape wine is grown in Slovenia. After several findings in all wine growing regions in the period from 2009 – 2012, the territory where majority of Slovenian vineyards are located (including all three wine-growing regions) was defined as the demarcated areas. Outbreaks were observed in Dolenjska region in the vicinity of Novo mesto (especially on the hybrid Noah) and in Primorska region in Slovenian Istria and in Kras on the variety Refošk.

After the first finding of GFD, the decision on eradication measures was adopted in 2006. Later in year 2009 the Regulation on prevention of spread and eradication of Grapevine flavescence dorée was accepted. The Regulation was amended in 2014 due to the reorganisation of the Administration (Phytosanitary Administration of the Republic of Slovenia was included together with the veterinary sector and food safety in new Administration for Food Safety, Veterinary Sector and Plant Protection).

The demarcated area is composed of a focus which is the territory around the infected plants with radius of up to 1 km. The focus is surrounded by the buffer zone which is at least 5 km wide.

Official measures are the following:

- in foci destruction of symptomatic plants and treatment of *Scaphoideus titanus* are obligatory,
- in buffer zone treatment of *Scaphoideus titanus* is obligatory and intensive survey is carried out by the Plant protection service. In case of suspicion of GFD phytoplasma, an official sample has to be taken for the laboratory analysis.
- in *Vitis* nurseries and nursery stocks, treatment of S. titanus is obligatory on the whole territory of Slovenia.

Detailed official measures for prevention of spread and eradication of the disease are defined in the Official action plan on control of Grapevine flavescence dorée.

Field monitoring of *S. titanus* in the wine growing region Posavje was carried out by the Plant protection service of Agricultural and Forestry Institute Novo mesto. Duration length of different developmental stages of *S. titanus* was investigated also in controlled conditions by Slovenian Institute of Hop Research and Brewing in Žalec.

In this paper the situation of Grapevine flavescence dorée phytoplasma in Slovenia is presented as well as the results of the monitoring of American grapevine leafhopper (*Scaphoideus titanus*) in the field and under controlled conditions.

MATERIAL AND METHODES

Monitoring including identification and separation of the developmental stages of *S. titanus* is crucial to determine optimal terms of treatment. *S. titanus* develops one generation per year. In its life cycle seven developmental stages are known: egg, two stages of larvae (L), three stages of nymphs (N) and imago.

Field monitoring

During the years 2012 - 2014, larvae hatching, their moulting periods and the occurrence of adults were monitored as well as the beginning and the top of occurrence of adults were monitored in the field. Monitoring was carried out on the location Zdole near Krško. Two methods were used: monitoring on leaves and monitoring on yellow sticky traps. On leaves mainly larvae and nymphs were recorded. Monitoring was carried out from the middle of May (approx. 15 May) until the occurrence of first adults, which are usually observed at the beginning of July. Monitoring of adults on yellow sticky traps was carried out from the beginning of July (app. 10 July) until the end of occurrence of adults (app. 5 October).

Controlled conditions

Duration length of different developmental stages was also investigated in controlled conditions. In growing chamber conditions hatching of leafhopper *S. titanus* larvae were assessed on the biennial shoots of grapevine which were collected in vineyard on location Zdole. The growing chamber was made by the manufacturer Kambič Laboratory Equipment d.o.o, Semič, Slovenia. Conditions in the camber: temperature 23 °C, 70 % relative humidity and 15-hour day length. 15 growing chambers were prepared and in each 10 two-year biennial shots with a length of 15 cm were put. The average

diameter of the shoots was 1,3 cm. At the bottom of the growing chambers vermiculite was put for maintenance of moisture. On the top of the chambers a filter paper was put and then the cut shoots were added. In every growing chamber a tube with water and young leaves of the variety Chardonnay were placed. Monitoring started on 29 March. Hatching of larvae in growing chambers was examined every two days, when new hatched larvae were counted. Then the larvae of American grapevine leafhopper were given separately in a Munger chamber with grapes leaves. Development of individual stages of *S. titanus* larvae continued under the same conditions as mentioned above.

RESULTS

Field monitoring

In the year 2012 first larva was observed on 25 May. The period of hatching lasted 34 days. Later on we observed first imago on the leaves on 4 July. First finding of imago on sticky yellow traps occurred on 18 July. The highest number of imagoes was recorded in the middle of August when 660 specimens in period of 14 days were caught. The catch this year was the highest (1407 adults were caught on this location). We finished the monitoring at the beginning of October.

Table 1. Results of field monitoring of American grapevine leafhopper in the year 2012

RESULTS – field monitoring (larvae and nymphs), year 2012	
First larva detected	25.5.
Period of hatching from egg to L1	25.5. – 28.6. (34 days)
Occurrence of L 2	14.6. – 4.7.
Occurrence of N 3	19.6. – 4.7.
Occurrence of N 4	28.6. – 18.7.
Occurrence of N 5	4.7. – 2.8.
Occurrence of IMAGO	4.7. – 7.10.
RESULTS – field monitoring (imago), year 2012	
First catch	18.7.
Highest catch	21.8., 660 adults
Total number of adults:	1407
End of catches	beginning of October

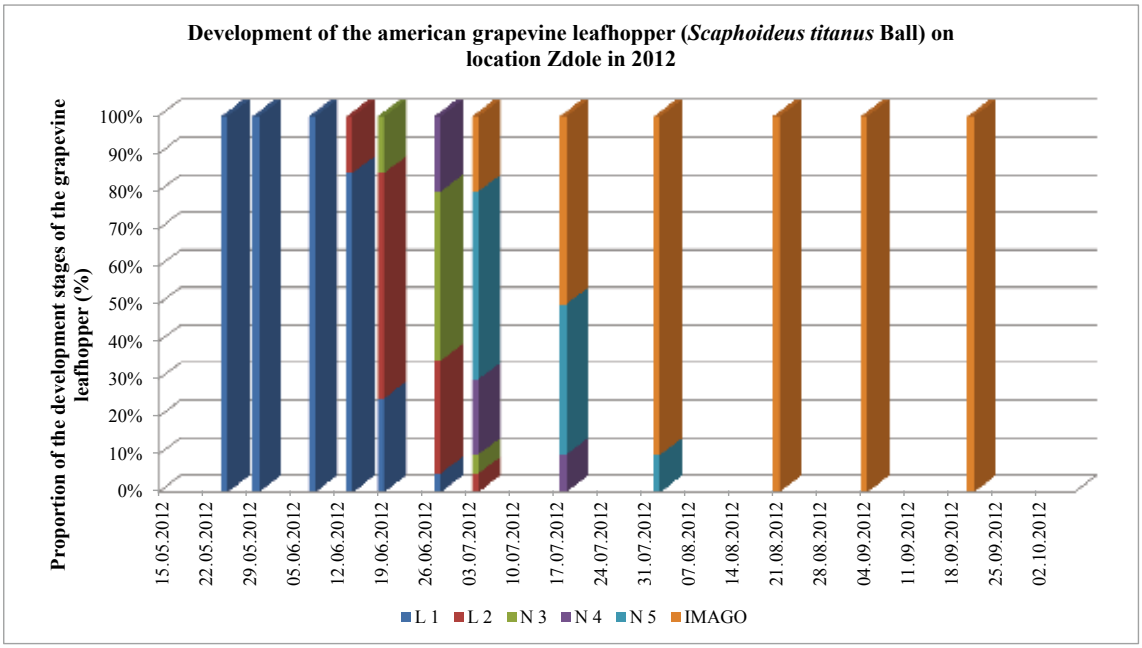

Figure 1. Development of the American grapevine leafhopper (*Scaphoideus titanus* Ball) on the location Zdole in 2012

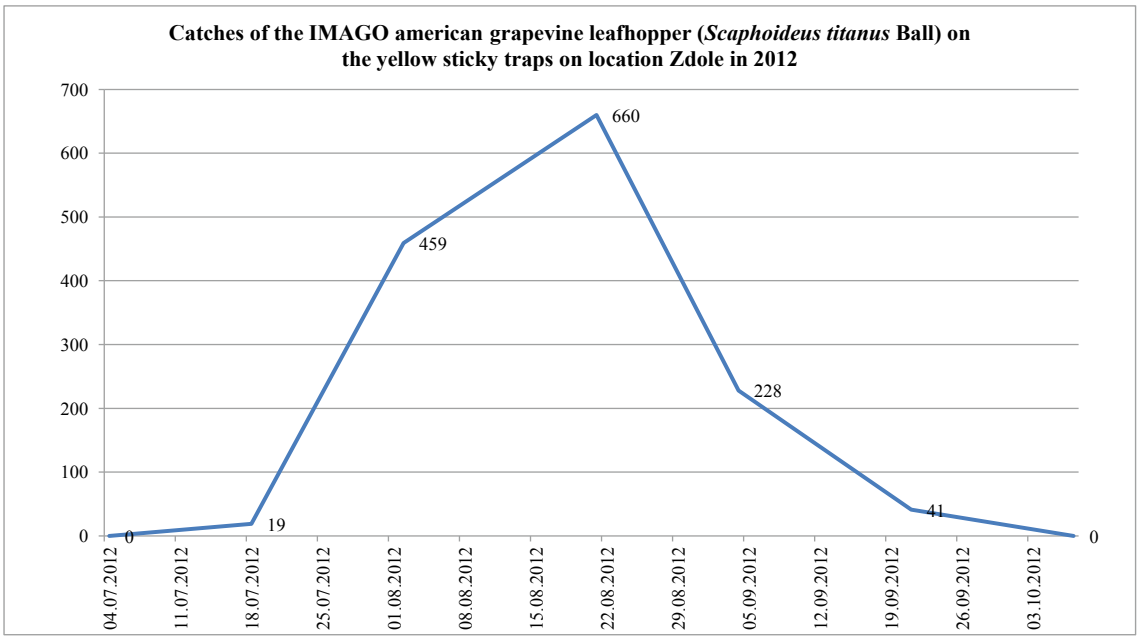

Figure 2. Catches of the IMAGO of American grapevine leafhopper (Scaphoideus titanus Ball) on yellow sticky traps on the location Zdole in 2012

In the year 2013 the first larva was detected on the leaves on 28 May, the period of hatching from eggs to stage L1 lasted 37 days. First imago on the leaves was observed in the middle of July and that the first catch on yellow sticky traps occurred on 23 July. In that year the highest number of imagoes was recorded at the beginning of August (232 adults). The highest catch in the year 2013 was 493 adults. We finished the monitoring in the middle of October.

Compared to the years 2012 and 2013, the beginning of larvae hatching was the earliest in year 2014 when also the period of hatching was the longest and lasted 38 days. Due to the weather conditions, the development was completed in 132 days and was the shortest in those 3 years.

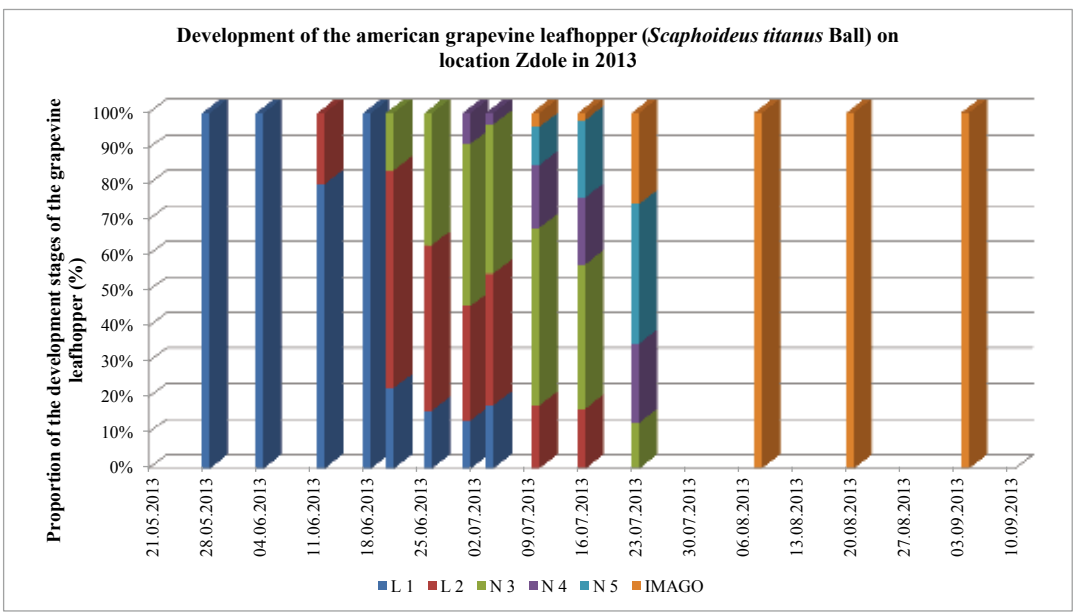

Figure 3. Development of the American grapevine leafhopper (*Scaphoideus titanus* Ball) on the location Zdole in 2013

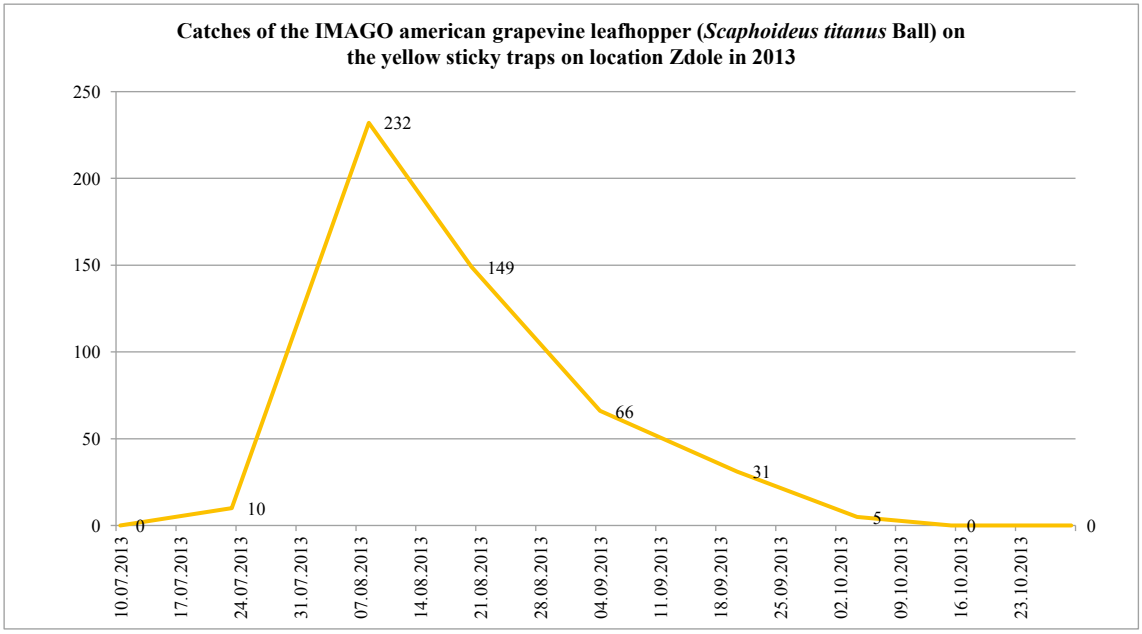

Figure 4. Catches of the IMAGO of American grapevine leafhopper (Scaphoideus titanus Ball) on yellow sticky traps on the location Zdole in 2013

Table 2. Results of field monitoring of American grapevine leafhopper in the year 2013

RESULTS – field monitoring (larvae and nymphs), year 2013	
First larva detected	28.5.
Period of hatching from egg to L1	28.5. – 4.7. (37 days)
Occurrence of L 2	12.6. – 16.7.
Occurrence of N 3	21.6. – 23.7.
Occurrence of N 4	1.7. – 23.7.
Occurrence of N 5	10.7. – 23.7.
Occurrence of IMAGO	10.7. – 10.9.
RESULTS – field monitoring (imago), year 2013	
First catch	23.7.
Highest catch	8.8., 232 adults
Total number of adults:	493
End of catches	middle of October

Table 3. Results of field monitoring of American grapevine leafhopper for the year 2014

RESULTS – field monitoring (larvae and nymphs), year 2014	
First larva detected	20.5.
Period of hatching from egg to L1	20.5. – 27.6. (38 days)
Occurrence of L 2	6.6. – 25.7.
Occurrence of N 3	18.6. – 25.7.
Occurrence of N 4	18.7. – 25.7.
Occurrence of N 5	18.7. – 25.7.
Occurrence of IMAGO	25.7. – 29.9.
RESULTS – field monitoring (imago), year 2014	
First catch	29.7.
Highest catch	11.8., 257 adults
Total number of adults:	459
End of catches	middle of September

The earliest larvae hatching was observed in the year 2014 (on 20 May) and in the same year we recorded the longest period of larvae hatching stage L 1 which lasted 38 days. The reason for such fast hatching in June 2014 may be the mild winter conditions. Sum of effective temperatures from 15 November to the beginning of hatching (threshold above 5°C) on the location Piršenbreg was 570 DD. This was the highest sum in these three years. The earliest occurrence of imago was recorded in the year 2012, in that year we caught the highest number of imagoes (660) within the period of 14 days. It is necessary to point out that in the years 2013 and 2014 the owner used insecticides for control of *S. titanus* and the consequence was decrease of the population.

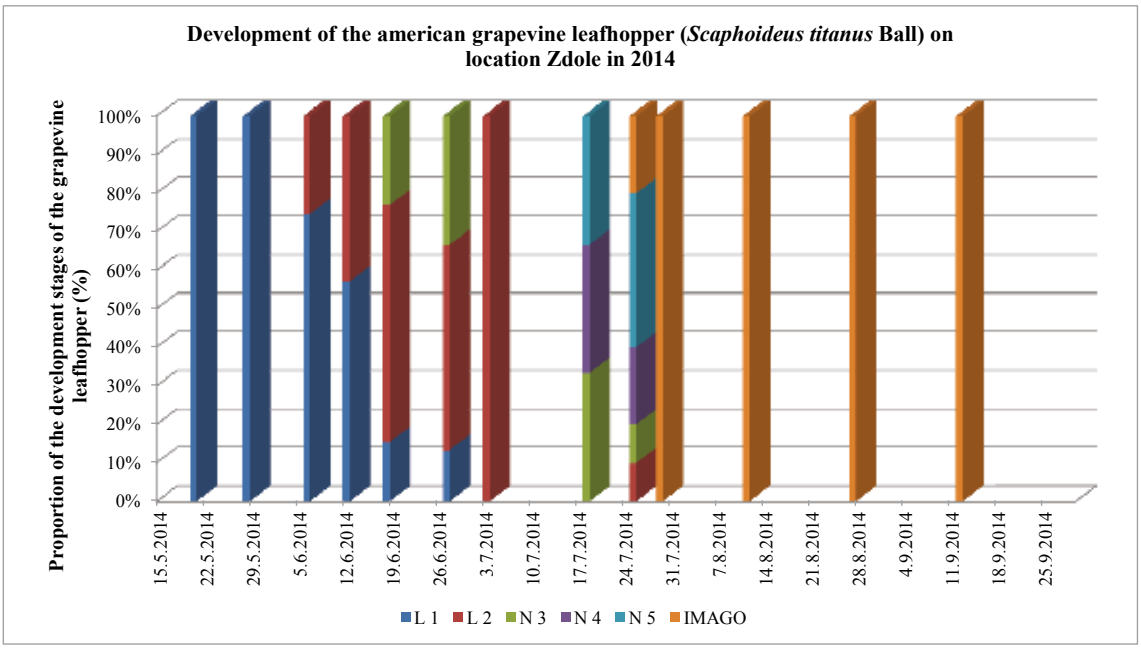

Figure 5. Development of the American grapevine leafhopper (*Scaphoideus titanus* Ball) on the location Zdole in 2014

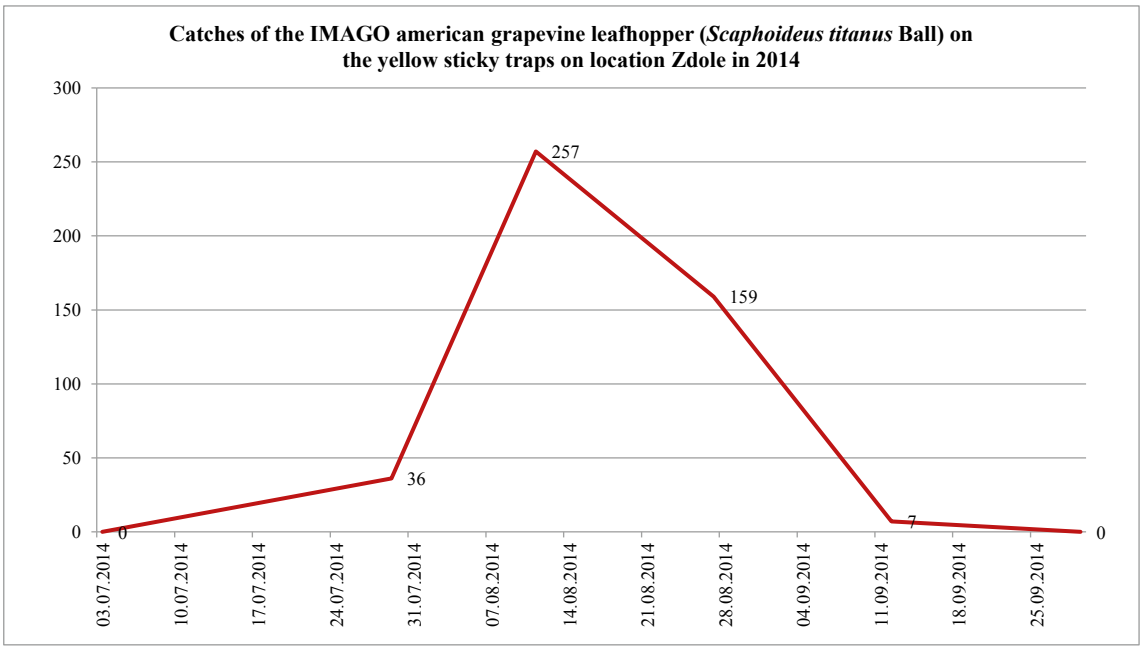

Figure 6. Catches of IMAGO of American grapevine leafhopper (Scaphoideus titanus Ball) on yellow sticky traps on the location Zdole in 2014

Controlled conditions

In Table 4 sums of temperatures are listed which are necessary for occurrence of first larvae in the growing chamber and in the field. Threshold of 5°C is used for calculation of the effective temperatures from 15 November until the beginning of first larvae hatching. The calculated average temperature of 592 DD is used as normative in determining the adequacy of temperature sums in the further research. Sum of temperatures are

also measured and calculated in the field (outdoors). The data collected from the ADCON agrometeorological stations serve as base. We can see that the measured temperatures in the field match with the average measured temperatures in the growing chambers.

Table 4. Sum of effective temperatures for occurrence of larvae L1 in growing chamber compared to the outdoors Zdole/Piršenbreg), treshold 5°C.

Year	Sum of effective temperatures (DD) above 5°C threshold when hatching of larvae starts (Zdole)
2010 – growing chamber	626
2011 – growing chamber	560
Average temp. in growing chamber	592
2012 – outdoors	569
2013 – outdoors	566
2014 – outdoors	570

Table 5. Sum of effective temperatures for development of larvae outdoors (Zdole/Piršenbreg), threshold 8,7°C (years 2012-2014)

Year	Sum of effective temperatures (DD) above threshold 8,7°C				
	L1	L2	N3	N4	N5
Average in laboratory ($DD_{8,7}$)	73	139	109	83	135
Sum in laboratory ($DD_{8,7}$)	73	212	321	404	539
2012 ($DD_{8,7}$)	164	73	123	98	347 ?
SUM 2012 ($DD_{8,7}$)	164	237	360	458	805 ?
2013 ($DD_{8,7}$)	98	131	85	111	154
SUM 2013 ($DD_{8,7}$)	98	229	314	425	579
2014 ($DD_{8,7}$)	69	63	133	310 ?	?
SUM 2014 ($DD_{8,7}$)	69	132	265	575 ?	?

When the first larvae are hatched we start to use threshold of 8,7°C. This threshold is used for calculation of degree days from the first larvae hatching until first occurrence of imago. From the year 2012 to 2014 the average temperature was calculated for each developmental stage and sum of temperatures from the beginning of hatching to the end of single stage. In the end we get sum of 539 DD. This temperature is used for comparison with other years. When the average temperature of 350 DD is reached, 30% of the N3 stage is hatched. This is the alarm to begin with the treatment with insecticides. In 2013, we see that the development outdoors is comparable to the calculated DD. The difference is approximately 30 DD which is around 3 days. The year 2014 on the basis of calculations and the actual situation outdoors showed that in the middle of July at the same time three development stages were present: N4, N5 and IMAGO.

DISCUSION

1. Temperature remains the main environmental factor in development of American grapevine leafhopper.

2. The research showed that low winter temperatures have an intensive influence on the beginning of larvae hatching.

3. Collected data on bionomy of American grapevine leafhopper were matched with the actual development in the vineyard. The measured sum temperatures in the growing chambers were comparable with the measured temperatures in the field.

4. Collected data present a good starting point for determination of regular term of treatment with insecticides in accordance with the Official action plan on control of Grapevine flavescence dorée in Slovenia.

The results in controlled conditions presented in this paper were obtained in the research work in professional Plant health task on Slovenian Institute of Hop Research and Brewing in Žalec and was founded by MAFF, Phitosanitary Administration of the Republic of Slovenia.

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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *Colletotrichum destructivum* FROM ALFALFA

Tanja Vasić [1], Vesna Krnjaja [2], Darko Jevremović [3], Snežana Anđelković [1], Dragan Terzić [1], Snežana Babić [1] and Dejan Šošić[5]

[1]Institute for Forage Crops, 37251 Kruševac, Serbia
[2]Institute for Animal Husbandry, Autoput 16, 11080 Belgrade, Serbia
[3]Fruit Research Institute, Kralja Petra I 9, 32000 Čačak, Serbia
[5]Magan Agrochemicals, 4/1 Petra Drapšina, 24000 Subotica, Serbia
e-mail: tanja.vasic@ikbks.com

ABSTRACT

The *Colletotrichum destructivum* O`Gara, which causes anthracnose disease in alfalfa (*Medicago sativa* L.) is an important limiting factor for alfalfa growth, plant vigor and persistence. In Serbia, the occurrence of anthracnose on alfalfa has been observed over the last several years (2005-2010). During the summer and autumn diseased plants start to appear in the field. *C. destructivum* was isolated from stem lesions typical of *C. trifolii*. The fungus colonizes infected stems and enters the crown and taproot, causing necrosis of tissue, predisposition to winter injury, and wilting or plant death. Isolates formed light green to dark olive-green colonies on PDA and developed black acervuli around the center of the colony. Conidia were hyaline, aseptate, and tapering at the one end and rounded at the other. Appressoria were smooth, simple, clavate to ovate, and varied from light to dark brown.PCR amplification (using universal primers ITS1/ITS4), produced a 495 bp amplicon for all tested strains. Primer pair GSF1-GSR1, yielded a 900 bp product and primers GDF1-GDR1 showed a 200 bp product for all strains examined. Based on the sequences of the PCR products of all isolates and morphological characteristics, the isolates from alfalfa were determined as *C. destructivum*.

Keywords: Anthracnose; Alfalfa; *Colletotrichum destructivum*; *C. trifolii;* PCR analysis

INTRODUCTION

Alfalfa is one of the most economically important perennial forage crops. Alfalfa provides first class fodder that is suitable for all live stock, especially ruminants. One of the reasons for smaller yield, thinning of alfalfa crops or a shorter period of exploitation, is inadequate resistance against the disease (Vučković, 1999). Many diseases cause the death of the plants, reduction of the yield, and impact alfalfa quality. Disease-causing agents attack specific parts or whole plants in various stages of growth and development (Vučković, 1999). Anthracnose of alfalfa is one of the most important diseases that reduce alfalfa yield (O'Neill et al., 1997, Mackie et al., 2003; Vasić, 2013) and is caused by fungi of the genus *Colletotrichum*. They are most commonly found in nature in the form of complexes and, as such, are probably involved in pathological processes causing mixed infections. The most important inducers of anthracnose of alfalfa in the world are *C. trifolii* and *C. destructivum* (Stuteville and Erwin, 1990; Mackie et al., 2003).

MATERIALS AND METHODS

Morphological traits of *C. destructivum* isolates

The isolates, studied in this paper, were obtained from diseased alfalfa plants collected from 2005-2010. Sampling was carried out in the main production areas of alfalfa in the territory of the Republic of Serbia. Collection of samples was performed after the first

cut during July, alfalfa was two years or older. All samples were placed in paper bags and delivered to the phytopathological laboratory of Institute for Forage Crops in Kruševac. The pathogen was isolated from the stem, the top of the root, and root of alfalfa. After bringing samples to the laboratory samples were first rinsed under running water and then, using standard methods, the isolation was performed. For the isolation of the pathogen, potato dextrose agar (PDA) medium was used (Dhingra and Sinclair, 1995). The Petri dishes were incubated in a temperature controlled incubator at 24 ± 2°C in the dark. This way, multiple isolates were obtained and for the further study six were selected based on the morphological traist and origin (Table 1).

Table 1. Summary of *Colletotrichum* spp. isolates selected for further study

Isolates	*District*	*Host*	Year
Coll-8	Raška	Alfalfa	2005
Coll-9	Rasina	Alfalfa	2005
Coll-18	Nišava	Alfalfa	2007
Coll-48	Pomoravlje	Alfalfa	2010
Coll-68	Pčinj	Alfalfa	2010
Coll-75	South Bačka	Alfalfa	2010
CC657	-	-	-

Morphological traits of selected isolates of *Colletotrichum* spp. were studied on PDA and CLA media (Waller et al. 1998), according to the method by Baxteret et al. (1983). The hyphae and reproductive structures of these parasites were observed using a compound light microscope. Hyphal appearance was performed according to the method by Baxteret et al. (1983) on PDA medium and in hanging drops over the glass according to the method described by Hawksworth (1974). The morphological traits of acervuli, specifically their appearance, size, and manner of their formation in cultures grown on PDA (Baxter et al., 1983) were studied. Size of conidiomata was determined by measuring the diameter (10 fully formed acervuli on PDA) and calculating the mean values. The presence or absence of setae in the cultures was determined by the method of Smith and Black (1990) by observation of 10-day-old cultures under the light microscope.The form and dimensions of conidia in the selected isolates of *Colletotrichum* spp. were examined according to the method of Smith and Black (1990). Thirty fully developed randomly selected conidia from 10 day-old-cultures were observed.Dimensions of conidia were determined by measuring the length and width of 30 randomly selected conidia in the selected isolates grown on PDA, using a light microscope (Olympus CX41) in total direct magnification 400x. Morphological traits of appresoria of the studied isolates were determined using a modified method by Hawksworth (1974). The shape, color and dimensions of appresoria were studied in six selected isolates of *C. destructivum*. Twenty-five appresoria per isolate were observed and measured. To observe the formation of teleomorph stage, six of the studied isolates were grown on PDA. Cultures were incubated at 25 °C in the day and night cycle, and the formation of perithecia was observed on three occasions, after 30 days, after 6 months and after 12 months. Petri dishes were kept in a temperature controlled incubator at a temperature of 25 °C. The trial was set to 10 repetitions per isolate.

MOLECULAR DETECTION AND IDENTIFICATION

Isolates were grown on PDA in the dark, at a temperature of 25 °C for 7 days. DNA extraction was done according to the method described by Day and Shattock (1997). For species-level determination of the *Colletotrichum* isolates. PCR was conducted using the three sets of primers: ITS1-ITS4 which amplifies the ITS region of rDNA Eucariota, GSF1-GSR1 which amplifies a portion of the glutamine synthetase gene (GS) and GDF1-GDR1 which amplifies a fragment containing an intron of the glyceraldehyde-3-phosphate dehydrogenase gene (GPDH). Table 2 lists the sequences of the primers and the expected amplicon sizes.

Table 2. Summary of primers used for the detection and identification of *Colletotrichum* spp.

Primers	*Sequences 5'-3'*	*Fragment length*	References
ITS1	TCCGTAGGTGAACCTGCGG	~495 bp	Freeman et al. (2000)
ITS4	TCCTCCGCTTATTGATATGC	~495 bp	Freeman et al. (2000)
GSF1	ATGGCCGATACATCTGG	~900 bp	Liu et al. (2007)
GSR1	GAACCGTCGAAGTTCCAC	~900 bp	Liu et al. (2007)
GDF1	GCCGTCAACGACCCCTTCATTGA	~200 bp	Liu et al. (2007)
GDR1	GGGTGGAGTCGTACTTGAGCATGT	~200 bp	Liu et al. (2007)

PCR amplification of the samples was performed in a TPersonal thermocycler (Biometra, Germany). The appearance of PCR products of the expected size was considered as a positive reaction. Analysis of the obtained PCR products was performed after electrophoretic separation of the resulting products in a 1.5% agarose gel. The 100 bp ladder (Amersham Biosciences, USA) was used in electrophoresis to determine the size of the products by comparison with the expected size of the DNA fragments. The amplified fragments in the gel were observed under UV light using a transilluminator (Biometra, USA). Identification of selected isolates was performed by multiple matching and calculation of the genetic relatedness of each of the obtained sequence, as well as with sequences of other isolates available in the GenBank database using the Blast analysis and Clustal W programs within the software package, the integrated MEGA 5.0.

RESULTS

Morphological traits of C. *destructivum* from Alfalfa

Isolates Coll-8, Coll-9, Coll-18, Coll-48, Coll-68, Coll-75 and CC657 grew 5 mm in diameter after the first day of plating. After the fifth day, the colonies reached 55 mm in diameter and the middle of the colonies started to darken. Edges of the colonies were slightly fibrous. The central part of the colony is whispy and velvety gray color to light olive green. The margin of the bottom side of the colony was light olive green while the center was a brighter olive green, with the beginnings of stromatic structures. All of the studied isolates of *C. destructivum* formed fruiting bodies - acervuli. The size of acervuli varied among the isolates from 100-280 μm. Acervuli within the colonies were grouped either in the central part, scattered or arranged concentrically.

In six selected isolates of *C. destructivum*, originated from alfalfa and grown in culture and compared with control strains of *C. destructivum* (CC657), it was found that setae were morphologically indistinguishable. In all the isolates, five days after plating numerous setae were formed within conidiomata, which are brown, with 1-7 septae, measuring from 50-150 x 2.5-7.5 μm.

Isolates Coll-8, Coll-9, Coll-18, Coll-48, Coll-68 and Coll-75 conidia were cylindrical, tapered at one end and curved at the other. According to conidial morphology, the isolates fit the description of conidia of *C. destructivum*. Conidia size in the studied isolates ranged from 10-25 x 2.5-7.5 μm. Also, the formation of septae in the equatorial part during conidial germination was determined for all of the isolates.

All of the *C. destructivum* isolates formed appresoria. Dimensions of appresoria ranged from 13.25-18.75 x 5.7-9.6 μm which were irregularly shaped, and dark brown in color.The *C. destructivum* isolates originating in Serbia as well as the reference CC657 isolate originating in Netherlands did not form perithecia in culture, , after 30 days, after 6 and 12 months to the complete exhaustion of cultures.

Molecular identification of *C. destrucivum* from Alfalfa

The polymerase chain reaction (PCR) has been successfully applied for the identification and characterization of six *C. destructivum* isolates obtained from alfalfa. Molecular detection of six selected isolates of *C. destructivum* originating from alfalfa, as well as reference strains of *C. destructivum* (CC657) was performed using universal primers ITS1/ITS4. The presence of fragments of the expected size of approximately 495 bp was observed in all isolates (Figure 1). The amplification did not occur in the negative controls.

Figure 1. Electrophoretic analysis of PCR products obtained using the primer pair ITS1/ITS4. Columns: 2 - isolate Coll-8, 5 - isolate Coll-9, 8 - isolate Coll-18, 13 - isolate Coll-48, 15 - isolate Coll-68, 16 - isolate Coll-75, 21 - reference isolate of *C. destructivum* (CC657), (-) - negative control, M - DNA Ladder Amersham Biosciences

Using the primer pair GSF1-GSR1 which enables amplification of the intron of the gene encoding glutamine synthetase (GS) and by comparing the

amplified fragments of the studied isolates tested with the marker (M), the presence of the amplicon of the expected size, of about 900bp was determined for all isolates (Figure 2).

Figure 2. Electrophoresis analysis of PCR products obtained using the primer pair GSF1-GSR1. Columns: 2 - isolate Coll-8, 6 - isolate Coll-9, 9 - isolate Coll-18, 14 - isolate Coll-48, 16 - isolate Coll-68, 17 - isolate Coll-75, 22 - reference isolate of C. destructivum (CC657), (-) - negative control, M-DNA Ladder Amersham Biosciences

Molecular detection of selected isolates was performed using the primer pair GDF1-GDR1 which amplifies ≈200 bp region of the glyceraldehyde-3-phosphate dehydrogenase (GPDH) intron. Using these primers, the intron for GPDH in all six isolates was successfully amplified (Figure 3).

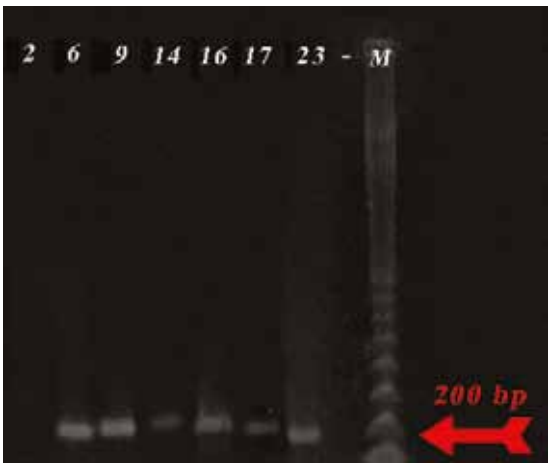

Figure 3. Electrophoresis analysis of PCR products obtained using the primer pair GDF1-GDR1. Columns: 2 - isolate Coll-8, 6 - isolate Coll-9, 9 - isolate Coll-18, 14 - Coll-48, 16 – isolate Coll-68, 17 - isolate Coll-75, 23 - referent isolate of *C. destructivum* (CC657), (-) - negative control, M - DNA Ladder Amersham Biosciences

DISCUSSION

Morphological traits of *C. destructivum* isolates

Comparative morphological studies were carried out with six isolates of *C. destructivum* which were the most similar to the reference isolate of *C. destructivum* (CC657). Isolates Coll-8, Coll-9, Coll-18, Coll-48, Coll-68 and Coll-75 were characterized as *C. destructivum* on PDA medium where they formed cottony colonies, velvety gray to light olive green in color. The results of these studies are similar to the results of other researchers (Baxter et al., 1983; Hyde et al. 2009), for *C. destructivum*.

All of the isolates of *C. destructivum* formed fruiting bodies - acervuli. Dimensions of acervuli ranged from 100-280 μm. Similar results were noted by Baxter et al. (1983) and Vasić (2013).The isolates formed setae within conidiomata, which were brown in color and were septate, having from 1 to 7 septae. Dimensions of setae were 50-150x2.5-7.5 μm. These results are similar to what was described by Baxter et al. (1983); Boland and Brochu (1989).

The isolates identified as *C. destructivum* formed conidia that were cylindrical, tapered at one end and rounded at the other, dimensions 10-25x2.5-7.5 μm. These results are similar to the results of other authors: Boland and Brochu (1989) Latunde-Dada and Lucas (2007); Frayssinet (2008).

In this study, it was found that the *C. destructivum* isolates formed septa in the equatorial part of the conidia during germination, which is a significant trait of this species. Based on this trait, *C. destructivum* differ from close related species which do not form septe. Latunde-Dada and Lucas (2007) i Shen et al. (2001) noted that the species *C. destructivum* forms septae during germination of conidia.

The studied isolates formed numerous appresoria, measuring from 13.25 to 18.75x5.7- 9.6 μm. The results obtained in this study coincide with the results of Latunde-Dada and Lucas (2007); Frayssinet (2008).

All of the *C. destructivum* isolates originated in Serbia, as well as control isolate CC657, originated in Netherlands, did not form perithecia, during the course of this experiment. These results are consistent with the finding from Baxter et al. (1983).

Molecular detection and identification of the anthracnose agent in alfalfa

The extracted fungal genomic DNA was intact and was suitable for PCR amplification, thus allowing for the successful detection and identification of all the isolates

selected for this study. By using different pairs of primers in performing PCR detection and identification, the differences in the specifics and suitability of detection of sequences of three different parts of the genome, ITS region, intron of the gene for GS and intron of gene for GPHD were determined.

Johnston and Jones (1997) used an analysis of LSU rDNA sequences of isolates derived from different crops in New Zealand. Moriwaki et al. (2002) successfully distinguished 236 isolates by studying ITS-2/LUS rDNA of *Colletotrichum* species in Japan using rDNA ITS1 region for sequencing products of size of approximately 157-190 bp. Freeman et al. (2000) have successfully distinguished 230 different isolates of *Colletotrichum* species using universal primers ITS1/ITS4 sequencing products of about 450-490 bp in all tested isolates.

Liu et al. (2007) stated that the use of intron regions in the glutamine synthetase (GS) gene of about 900 bp has proved successful for the identification and characterization of closely related species within the genus *Colletotrichum* which were previously difficult to distinguish based on morphological traits. This research showed that the intron region of the gene for GS is suitable for the identification of *C. destructivum*.

Detection and identification of the isolates was carried out using the GDF1/GDR1 primers that allow the amplification of a portion of the second intron of the glyceraldehyde-3-phosphate dehydrogenase (GPHD). These primers produced fragments of about 200 bp in all tested isolates. The first multilocus phylogenetic analysis of the genus *Colletrichum* was done by Talhinhas et al. (2002) by studying *C. acutatum* community on lupine (*Lupinus*) using ITS, TUB2 and HIS4 sequences. Guerber et al. (2003) used the intron region of a gene for GS and GPHD nucleotide sequences for the analysis of *C. acutatum*.

As populations of *C. destructivum* in alfalfa from Serbia has not been studied at the molecular level so far, their genetic structure is not known, as well as the variability in the Serbian isolates compared to isolates of *C. destructivum* originating from Europe and other parts of the world. These results represent the first detailed characterization of this species in Serbia.

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RACE DIFFERENTIATION WITHIN STRAINS OF *Xanthomonas euvesicatoria* CAUSAL AGENT OF BACTERIAL SPOT OF PEPPER IN SERBIA

Maja Ignjatov[1], Milan Šević[2], Jelica Gvozdanović-Varga[1], Katarina Gašić[3], Dragana Milošević[1] and Aleksa Obradović[4]

[1] Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia
[2] Institute of Vegetable Crops, Karađorđeva 71, Smederevska Palanka
[3] Institute for Plant Protection and Environment, Teodora Drajzera 9, 11040 Belgrade, Serbia
[4] University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade, Serbia
maja.ignjatov@nsseme.com

ABSTRACT

Bacterial spot of pepper, caused by *Xanthomonas euvesicatoria* regularly causes losses in pepper production in Serbia. During 2008, 2009 and 2010 samples of diseased pepper leaves with bacterial spot symptoms were collected from different localities in Serbia. Total of 116 strains of bacteria were obtained by isolation from infected leaves. Within the world population of the pathogen 11 physiological races are distinguished on the basis of reaction on pepper variety ECW and their isogenic lines known as ECW10R (*Bs1* gene), ECW20R (*Bs2* gene), ECW30R (*Bs3* gene) and PI 235047 (*Capsicum pubescens*). Race differentiation of Serbian *X. euvesicatoria* strains was carried out based on the reaction of differential plants. Our studies showed that the population of *X. euvesicatoria* was heterogeneous, consisting of four physiological races: P1, P3, P7 and P8. The most common was the pepper race P8, followed by P7, P1 and P3 represented by the 93, 17, 5 and 1 strain, respectively.

Key words: pepper race, bacterial spot, *Xanthomonas euvesicatoria*

INTRODUCTION

According to the latest classification, bacterial spot (BS) of pepper could be caused by three species of Gram-negative bacteria belonging to the genus *Xanthomonas*: *X. euvesicatoria*, *X. vesicatoria* and *X. gardneri* (Vauterin *et al*., 1995; Stall *et al*., 1994; Jones *et al*., 2004; Obradović *et al*., 2004; Bull *et al*., 2010). The pathogen is seed-borne, but from season to season it can persist in the field on weeds, volunteer plants and debris of infected plants (Jones *et al*., 1986; Obradović *et al*., 2008). Under humid and warm weather conditions, bacterial spot of pepper, caused by *Xanthomonas euvesicatoria* regularly causes losses in pepper production in Serbia (Obradović *et al*. 2000a, 2004; Ignjatov *et al*. 2010). Susceptibility of pepper varieties grown in Serbia certainly contributes to frequent occurrence of bacterial spot. Currently, 11 physiological races of *X. euvesicatoria* are distinguished based on the reaction on pepper variety ECW and its isogenic lines ECW10R (*Bs1* gene), ECW20R (*Bs2* gene), ECW30R (*Bs3* gene) and PI 235047 (*Capsicum pubescens*) (Stall *et al*., 2009) (tab. 1). The aim of this study was to differentiate races within strains of *X. euvesicatoria* collected from different pepper production regions in Serbia.

MATERIAL AND METHOD

A three-year survey (2008-2010) of pepper fields resulted in isolation of numerous bacterial strains. After obtaining pure cultures 116 strains were chosen for

further study. Pepper plants of Early Calwonder (ECW), and differential isogenic lines were grown for 3-4 weeks in a growth chamber until the fourth true leaf was fully expanded. Infiltration of a leaf is accomplished by gently forcing the bacterial suspension (10^8CFU/ml) into the underside of the leaf using a sterile needless syringe. Plants were incubated in the laboratory at 22-24 °C. Rapid collapse of the infiltrated area, followed by necrosis within 24h, was considered hypersensitive reaction (HR). Development of water-soaked lesions after 3-5 days indicated a susceptible reaction (C).

RESULTS

According to the reaction of differential isogenic lines, our studies showed that the population of *X. euvesicatoria* was heterogeneous with four physiological races present: P1, P3, P7 and P8. The most common was the pepper race P8, followed by P7, P1 and P3 represented by the 93, 17, 5 and 1 strain, respectively. The pepper isogenic line ECW-20R, carrying *Bs2* resistant gene, reacted hypersensitively to all investigated strains. Race composition of pepper strains and presence of four races (P1, P3, P7, P8) indicated that introduction of *Bs2* gene in commercial varieties would control resistance to majority of the strains included in this study. Susceptible reaction appeared 3-5 days after infiltration as chlorotic, water soaked tissue within the infiltrated area (Fig. 1a). Resistant reaction can vary in appearance from bleached white with a dark border to uniformly dark brown color throughout the infiltrated, collapsed area (Fig. 1b).

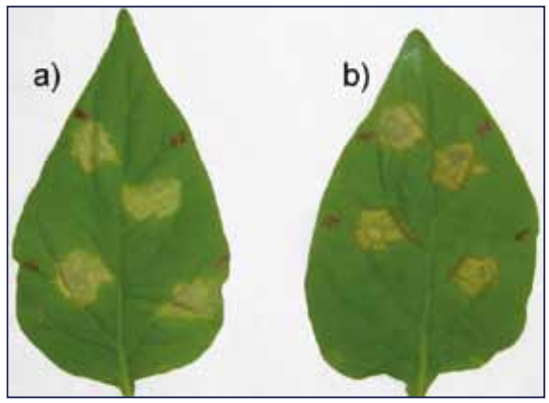

Fig. 1. Reaction of differential pepper lines: a) compatible (susceptible) reaction (C) on pepper cv. ECW; b) resistant reaction (HR) on pepper cv. ECW20

DISCUSSION

Bacterial spot of pepper caused by *X. euvesicatoria* occurs regularly in Serbia, causing significant losses due to reduction of leaf area, defoliation and fruit scab (Obradović *et al*., 1999, 2000b; Ignjatov *et al*., 2010; Gašić *et al*., 2011). Breeding programmes for BS-resistance are considered as one of the most effective strategies for controlling the disease. However, the development of resistance has been limited by the high degree of genetic and phenotypic diversity within the *Xanthomonas* species complex. Over the last 20 years, the pathogen distribution and diversity was observed in Serbia and the presence of pepper races 1, 3, 7 and 8 was already reported (Obradović *et al*., 2000a, 2004).

Table 1. Differentiation of *X. euvesicatoria* races using known resistance genes in pepper (Stall *et al*., 2009)

Races	Funcional avirulence gene (*avr*)	Pepper diferential lines				
		ECW	ECW-10 *Bs1*	ECW-20 *Bs2*	ECW-30 *Bs3*	PI235047 *Bs4*
P0	*avrBs1*, *avrBs2*, *avrBs3*, *avrBs4*	C	HR	HR	HR	HR
P1	*avrBs2*, *avrBs3*, *avrBs4*	C	S	HR	HR	HR
P2	*avrBs1*, *avrBs2*	C	HR	HR	C	C
P3	*avrBs2*, *avrBs4*	C	C	HR	C	HR
P4	*avrBs3*, *avrBs4*	C	C	C	HR	HR
P5	*avrBs1*	C	HR	C	C	C
P6	*avrBs4*	C	C	C	C	HR
P7	*avrBs2*, *avrBs3*	C	C	HR	HR	C
P8	*avrBs2*	C	C	HR	C	C
P9	*avrBs3*	C	C	C	HR	C
P10	nema	C	C	C	C	C

ECW - Early Calwonder (No R gene); C – compatible (susceptible) reaction; HR - hypersensitive (resistant) reaction; PI235047-*Capsicum pubescens*

First, the pepper races P1 and P3 were reported (Obradović *et al*., 2008), later after *Bs4* gene were identified in *Capsicum pubescens* as PI235047 (Sahin i Miller, 1998), new pepper races of *X. c.* pv. *vesicatoria* (P7, P8) were differentiated in Serbia (Obradović *et al*. 2004). Results of the pepper differential cultivars presented in this study showed that the population of Serbian strains of *X. euvesicatoria* still consists of four races: P1, P3, P7 and P8. The results indicated that the race P8 was predominant in Serbia during all three years of survey. The pepper isogenic line ECW-20R, with *Bs2* resistant gene, reacted hypersensitively to all investigated strains. As source of resistance to race 8 mostly present in Serbia, introduction of *Bs2* into commercial pepper genotypes would provide better control of the pathogen. Since pepper is intensivly grown in Eastern Europe and in Mediterranean countries, we can not ignore the possibility of introduction of new races.

In Europe the different pepper races were determined in Italy, Hungary, Romania and in Eastern Meditarrean region of Turkey (Bouzar *et al*. 1994; Buonaurio *et al*., 1994; Sahin, 2001). Mitrev and Kovačević (2006) reported that pepper races P0 and P2 were predominant in Republic of Macedonia. Selection pressure created by entering the resistance genes in commercial genotypes often results in the appearance of strains able to overcome resistance (Pernezny and Collins, 1997). Therefore, determination of the genetic diversity of local pepper affecting *Xanthomonas* population is a prerequisite for the development of durable resistance to BS (Stall *et al*. 2009).

CONCLUSION

Race composition of *X. euvesicatoria* strains and presence of four races (1, 3, 7, 8), with prevailing race P8, indicate that introduction of *Bs2* gene would be effective against the majority of strains found in this study. In conclusion, the results presented here defined the target for breeding programs and creating resistant lines and varieties of pepper. Host-plant resistance is an effective method for plant disease management.

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OCCURRENCE OF GRASS BUNT IN VOJVODINA AND ITS INFLUENCE ON WHEAT SEED QUALITY CONTROL

Vesna Župunski and Radivoje Jevtić

Institute of Field and Vegetable Crops
Maksima Gorkog 30, 21000 Novi Sad, Serbia
e-mail: vesna.zupunski@nsseme.com

ABSTRACT

Causal agents of grass bunt, which infect wild grass hosts in *Bromus, Festuca, Ventenata* and *Vulpia,* are *Tilletia* species whose teliospores could be found in wheat seed samples too**.** Among them, *T. bromi* is morphologically similar to those of quarantined *T. contraversa,* and misidentification of this species could lead to commodity rejection. As a result, monitoring the presence of teliospores of quarantined species in seed samples, and identification of *Tilletia* species on the basis of morphological characteristics is difficult and in some cases impossible. In order to investigate the presence of grass bunt in seed samples of wheat in Vojvodina, teliospore extraction was carried out by using the size-selective sieving wash method and the OEPP/EPPO diagnostic protocol for *Tilletia indica* (2007). The analysis of 151 samples of basic, certified and commercial non-processed seed of wheat revealed that 127 samples were contaminated with *T. caries*, while 12 samples were contaminated with teliospores of *Tilletia* species which had morphological characteristics that correspond to *T. bromi* complex or quarantined *T. contraversa*. These teliospores displayed prominent gelatinous sheath with conspicuous depth of reticulations. Molecular identification of grass bunt teliospores was not possible in this study, because contamination level was too low (1 teliospore per 10 seeds). However, knowing that teliospores of *T. bromi* often occur in wheat seed samples in very low numbers there is a need for standardization of molecular techniques for the identification of a single teliospore of *Tillletia* species in order to make plant protection more efficient and reliable.

Key words: grass bunt, *Tilletia bromi, Tilletia contraversa,* seed testing

INTRODUCTION

The genus *Tilletia* includes approximately 100 known species that infect grass hosts (Pimentel et al., 2000). Economically important pathogens of wheat (*Triticum aestivum* L.) are *Tilletia caries* (DC.) Tul. & C. Tul. and *T. foetida* (Wallr.) Liro causal agents of common bunt, as well as *T. contraversa* J.G. Kühn causal agent of dwarf bunt (Goates, 2012). In addition, there are *Tilletia* species whose teliospores could also be found in samples of wheat seed, although it is known that wheat is not a host. Representatives of *T. bromi* (Brockm.) Brockm complex (*T. bromi-tectorum* and *T. guyotiana*), are causal agents of grass bunt, and infect wild grass hosts in *Bromus, Festuca, Ventenata* and *Vulpia*. The presence of teliospores of *T.bromi* in wheat seed samples could make quarantine inspection very difficult (Peterson et al., 2009) because of morphological similarity of teliospores. As a result, investigation of *T. bromi* has become increasingly important.

Identification of *Tilletia* species using light microscopy is almost impossible because of overlapping of morphological characteristics of teliospores (Mathre, 1996; Peterson et al.,2009). According to Goates (1996) sheath thickness of *T. contraversa* is 1.5 to 5 μm, and depth of reticulations is 1.5 to 3 μm. Boyd and Carris (1998) reported that depth of reticulations and sheath thickness of *T. bromi* is 1 to 3 μm, thus teliospores with sheath

thickness between 1 and 3 μm could be identified as *T. bromi* as well as *T. controversa*. Although identification of *Tilletia* species is done using areolae diameter, it is not discriminative enough. Goates (1996) reported that diameter of areola for *T. contraversa* is 3 to 5 μm, while Boyd and Carris (1998) reported areolae diameter 2 – 5 - (7) μm for *T. bromi*. Trione and Krygier (1977) noted that average sheath thickness and depth of reticulations of *T. bromi-tectorum* and *T. guyotiana* are 1.54 μm and 2.52 μm, respectively, which was in accordance with results reported by Boyd and Carris (1998).

Common and dwarf bunt have been successfully controlled by using combination of fungicides and resistant cultivars, however, European agriculture has been moving toward organic production and low-input farming systems (Matanguihan et al., 2011). As consequence, the need for monitoring the presence of *Tilletia* species, varieties and races in wheat production areas is growing (Jevtic, 1998). In addition, *T. contraversa* is still quarantined species in number of countries including Republic of Serbia. As a result, investigation of presence of *Tilletia* species in wheat production areas in Vojvodina, with special reference to the presence of grass bunt in seed samples of wheat is conducted as part of the program of establishment and implementation of international standards for phytosanitary field.

MATERIAL AND METHODS

Autumn-sown wheat samples were collected in cooperation with the regional phytosanitary laboratory Agroinstitut-Sombor, during the 2007-2008. In total, 151 non-processed seed samples of 1 to 2 kg were sampled from trucks. Seed samples were stored at 15°C and relative humidity of< 60% (Elias et al., 2007). In order to investigate the presence of grass bunt in seed samples of wheat in Vojvodina, teliospore extraction were carried out by using the size-selective sieving wash method described by Peterson et al. (2000) and modified OEPP/EPPO diagnostic protocol for *Tilletia indica* (2007). Teliospores were extracted from 50 g subsamples of each grain sample using 10 μm nylon mesh. Extracted teliospores were suspended with 100 μl (or more) of 15% glycerol, depending on the pellet volume (OEPP/EPPO, 2007). If teliospores were not found in the first 50 g subsample, two further 50 g subsamples were examined in order to determine the presence of *Tilletia* species with confidence level of 99% (OEPP/EPPO, 2007). Identification of extracted teliospores was processed using light microscopy at 630× magnification on the bases of morphological characteristics of *Tilletia* species reported by Goates (1996) and Boyd and Carris (1998). Contamination level was determined by calculating total number of teliospores per 50 g seed subsample, with the use of the Breed Method.

RESULTS

The analysis of 151 samples of basic, certified and commercial non-processed seed of wheat revealed that 12 samples were contaminated with teliospores of *Tilletia* species morphologically similar to quarantined *T. contraversa* and *T. bromi*. These teliospores displayed prominent gelatinous sheath with conspicuous depth of reticulations, which made them different from the most dominant *T. caries*. Diameter of grass bunt teliospores was 18 – 22 μm, and that characteristic was not used for making distinction between *T. contraversa* and *T. bromi* since teliospore diameters of those species are 16.8 - 32 μm and 18 – 29 μm, respectively. Taking into account that areolae diameter ranged from 2 to 4 μm, only teliospores with areole diameter lesser than 3 μm were assumed to belong to *T. bromi* (2 – 7 μm) since areolae diameter of *T. controversa* is 3 - 5 μm. Sheath thickness of newly found teliospores ranged from 1.5 to 3.5 μm, which overlaps with sheath thickness of *T. contraversa* (1.5 – 5 μm) and *T. bromi* (1 - 3μm). Morphological characteristics of newly found teliospores of *Tilletia* spp. are presented in Table 1 and Figure 1.

Teliospores of grass bunt were found in seed samples which were contaminated with less than 100 teliospores per 50 g subsample, that is, one teliospore per 10 seeds, thus it was not possible to make identification using molecular technique regarding difficulties in production of mycelal mats for DNA extraction.

Table 1. Morphological characteristics of teliospores of *Tilletia* spp.

Teliospore	Areolae diameter		Sheath thickness		Identification
	T. contravesa 3 - 5μm	*T. bromi* 2 – 5- (7) μm	*T. contraversa* 1.5 – 5 μm	*T. bromi* 1 – 2.53 (3) μm	
a	-	2.67 – 2.83	1.95 – 3.39	-	Non- identified
b	-	2.73 – 3.80	1.56 – 2.56	1.56 – 2.56	*T. bromi*
c	3.55 – 3.77	3.55 – 3.77	2.49 – 2.92	2.49 – 2.92	*T. bromi / T. contraversa*

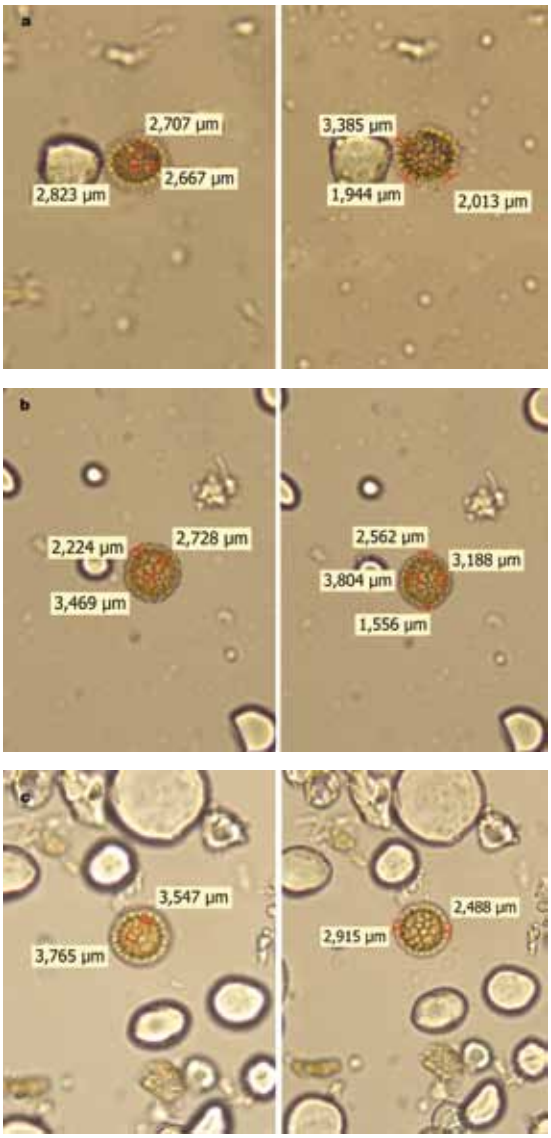

Figure 1. Teliospores of *Tilletia* spp. with prominent gelatinous sheath

a – Non-identified teliopsore
b – Teliospore identified as *T. bromi*
c – Teliospore that could be identified as either *T. bromi* or *T. contraversa*

DISCUSSION

Occurrence of teliospores of *Tilletia* spp. causal agents of grass bunt in wheat seed samples has been reported by many authors (Peterson et al., 2000; Pimentel et al., 2000; Trione and Krygier, 1977). The presence of teliospores of *T. bromi* in wheat seed samples could be a problem for commodity inspection due to teliospore similarity with *T. contraversa*. As a result, seed testing for the presence of *Tilletia* species is almost impossible when it is based only on morphological characteristics of teliospores (Goates, 1996, Mathre, 1996; Peterson et al., 2009). In this study, areolae diameter and sheath thickness were not discriminative enough for making distinction between *T. conraversa* and *T. bromi* because of overlapping of morphological characteristics. Majority of teliospores could be identified as either *T. contraversa* or *T.bromi*.

In addition, there is a rising question about measurement uncertainty, especially in cases when measured values reach the limit separating one species from the other. Areole diameter of teliospore marked as ***a*** (Fig 1) was measured to be 2.83 µm which is very close to the limit of 3 µm used to discriminate *T. bromi* from *T. contraversa*. As a result, teliospore marked as ***a*** could not be identified as neither *T. bromi* nor *T. contraversa*.

Inability to identify single teliospore in seed samples using morphological characteristics could make not only quarantine decisions difficult, but also decisions about intervention in organic agriculture where determination of exact teliospore number is of crucial importance. For example, prescribed teliospore thresholds for intervention in organic agriculture vary in different countries and it is 20 common bunt teliospore/seed in Germany, 10 teliospores/seed in Austria and Switzerland and one teliospore/seed in the United Kingdom (Matanguihan et al., 2011). Contamination limits for *T. contraversa* in Austria, Switzerland and Scotland are the same as the previously mentioned (Micheloni et al., 2007). However, in Denmark, intervention is recommended at the first detection of teliospores (Matanguihan, 2011).

In order to overcome the problem related to identification of *Tilletia* species many different molecular techniques were used. Initially, genomic fingerprinting techniques were good enough to make distinction between wheat and grass bunt fungi, but not precise enough in distinguishing *T. contraversa* from *T. caries*. Usually, they were processed by using molecular markers such as RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and PCR-RFLP (restriction fragment length polymorphism) of ITS rDNA region. (Boyd et al., 1998, Pimentel et al., 1998; Shi et al., 1996). Later, species specific primers were selected for making distinction between *T. contraversa* and *T. caries*, however it has not be confirmed that those primers could make distinction between *T. contraversa* and *T. bromi* (Yuan et al., 2009; Liu et al., 2009; Gao et al., 2010; Gao et al., 2011). Finally, rep-PCR fingerprinting technique, with REP, ERIC and BOX primers, succeeded in making distinction not only between *T. contraversa* and *T. caries* but also

between *T. contraversa* and *T. bromi* (McDonald et al., 2000). Župunski et al. (2011) confirmed results obtained by McDonald et al. (2000), and reported coefficients of similarities between *Tilletia* species which were in accordance to those reported by McDonald et al. (2000).

In this study, molecular identification of grass bunt teliospores was not possible since contamination level was too low (1 teliospore per 10 seeds). Župunski et al. (2011) succeeded in application of rep-PCR fingerprinting for *Tilletia* species identification only when contamination level was 1 teliospore per 1 seed or higher. Molecular techniques such as real-time PCR and multiplex real-time PCR techniques was successful in quantifying bunt contamination levels down to less than one spore per seed, but they were not successful in distinguishing *T. caries, T. contraversa* and *T. bromi* (McNeil et al.,2004; Tan et al.,2009). McDonald et al. (1999) also tried to make identification of various *Tilletia* species using single ungerminated teliospore but test sensitivity varied for the different *Tilletia* species and different teliospore lots. Knowing that teliospores of *T. bromi* often occur in wheat seed samples in very low numbers and that all molecular techniques which were successful in distinguishing *Tilletia* species were established using large number of teliospores (Gao et al., 2010, 2011; Kellerer et al., 2006; Liu et al., 2009; McDonald et al., 2000; Yuan et al., 2009), there is a growing need for validation of these methods on wheat seed samples with low levels of contamination. Standardization of molecular techniques for the identification of a single ungerminated teliospore of *Tillletia* species will contribute not only in testing for the presence of quarantined species but also in investigations where extraction of DNA from germinated teliospores is the great obstacle.

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WEED FLORA OF VINEYARD IN BOSNIA AND HERZEGOVINA

Zlatan Kovačević, Biljana Kelečević and Siniša Mitrić

University of Banja Luka, Faculty of Agriculture
Bulevar vojvode Petra Bojovica 1 A, 78000 Banja Luka, Bosnia and Herzegovina

ABSTRACT

Two-year study (2008-2010) weed flora of vineyards in Bosnia and Herzegovina (B&H) performed on 51 locality. As result of this research it was found 133 species of vascular plants covered with: 112 genera, 39 families, 4 class and 2 divisions. The analysis of the biological spectrum showed 5 life forms with predominant presence of terophytes (45.86%), hemicryptophytes (39.85%) and geophytes (9.77%). Phytogeography analysis has been allocated 9 floristic groups, and the most common are: Cosmopolitan, Eurasian, Mediterranean, Boreal, Adventive and sub-Mediterranean, and together comprise 125 species (93.98%). It is very significant participation of 14 adventive species, and some species have taken invasive character, for example *Ambrosia artemisiifolia* L. Weed flora of vineyard in B&H is rich in flora due to the existence of continental and sub-Mediterranean wine-growing region. Considerable diversity is caused by the specifics of the study area, which are reflected in different climatic, edaphic and orographic characteristics, plant-geography, and different intensities of anthropogenic influences, traditions and the cultivation of grapevine. On the other hand it is important a presence of cosmopolitan and adventive species that are more or less extensively spread, and beside of typical weed and weed-ruderal species in weed flora of vineyards in B&H it was determined a significant number of ruderal and meadow species.

Key words: weed flora, vineyard, Bosnia and Herzegovina.

INTRODUCTION

Studies of weed flora in vineyards of B&H is related to weeds that form anthropogenic plant communities that are, unlike natural plant communities, characterized by the fact that in their forming, structure and development crucial importance has anthropogenic factor. Agrophytocenoses of vineyards are relatively unstable formations and if we exclude impact of anthropogenic factor, they return to the climax communities that typically have a zonal character. By analysis of the weed flora, in indirect way, it is possible to examine the habitat requirements of the study area, the impact of environmental characteristics of the habitat and human influence on the composition and properties of weed flora. Diversity of weed flora in vineyards of B&H caused by the specifics of study area, which are reflected in different climatic, edaphic and orographic characteristics, plant-geography, and different intensities of anthropogenic influences, traditions and systems of grape vine growing.

MATERIAL AND METHODS

Floristic research of weed flora of vineyards were conducted during two growing seasons and included the 51 site. The determination was based on publications: Flora Europaea IV (Tutin, ed., 1964-1980), Flora of SR Serbia I-IX (Josifović, ed., 1970-1977) and Flora of Croatia (Domac, 1994). Taxonomy and nomenclature is conforms to the publication of Flora Europaea IV (Tutin, ed., 1964-1980). Life forms of plants were determined according to the amended classification given in the Flora of Serbia (Sarić, ed., 1992). Reference of floral elements was determined by Oberdorfer (2001), (based on areal maps Meusel et al. and Atlas flora of Europe) that contain the original range of cosmopolitan and adventive species.

RESULTS

In the table 1 it is given an overview of the identified species vascular weed flora of vineyards in B&H.

Tab. 1. Overview of the identified species of vascular weed flora of vineyards in B&H

Plant species	Life forms[1]	Floral elements[2]
1	2	3
Achillea millefolium L.	H	bor-euroas.suboc
Agropyron repens (L.) Beauv.	G	cosm
Agrostis stolonifera L.	H	cosm
Ajuga reptans L.	H	subatl-smed
Alchemilla vulgaris L.	H	bor
Allium vineale L.	G	subatl-smed
Amaranthus albus L.	T	adv
Amaranthus retroflexus L.	T	adv
Ambrosia aretemisiifolia L.	T	adv
Anagallis arvensis L.	T	cosm
Anthemis arvensis L.	T/H	euroas.suboc-med
Aristolochia clematitis L.	G	smed
Asclepias syriaca L.	G	adv
Avena barbata Pott. ex Link.	T	adv
Bellis perennis L.	H	subatl-smed
Berteroa mutabilis (Vent.) DC.	T	cont
Bidens bipinnata L.	T/SH	adv
Bidens tripartita L.	T	euroas-smed
Bilderdykia convolvulus (L.) Dumort.	G	cosm
Bromus hordeaceus L.	T	cosm
Calystegia sepium (L.) R.Br.	SH	cosm
Campanula patula L.	H	euroas
Capsella bursa-pastoris (L.) Med.	T/H	cosm
Centaurium erythraea Rafn.	T/H	circ
Chenopodium album L.	T	bor-euroas
Chenopodium polyspermum L.	T	euroas.suboc
Chondrilla juncea L.	T/H	med-smed-cont
Cichorium intybus L.	H	cosm
Cirsium arvense (L.) Scop.	G	bor-euroas.smed
Clematis flammula L.	S	med-smed
Consolida regalis S.F.Gray	T	euroas-smed
Convolvulus arvensis L.	G	cosm
Conyza canadensis (L.) Cronq.	T	adv
Crepis biennis L.	H	mod.cont
Crepis sancta (L.) Babcock	T/H	med
Cynodon dactylon (L.) Pers.	G	cosm
Dactylis glomerata L.	H	euroas.suboc-smed
Datura stramonium L.	T	cosm
Daucus carota L.	H/T	euroas.suboc-smed
Digitaria sanguinalis (L.) Scop.	T	circ
Diplotaxis muralis (L.) DC.	T/H	med-smed
Echinochloa crus-galli (L.) Beauv.	T	cosm
Echium italicum L.	H	med-atl
Equisetum arvense L.	G	circ
Erigeron annuus (L.) Pers.	T/H	adv
Euphorbia chamaesyce L.	T	med
Euphorbia helioscopia L.	T	cosm
Foeniculum vulgare Mill.	H	adv
Fumaria officinalis L.	T	euroas.suboc
Galinsoga parviflora Cav.	T	adv
Galium mollugo L.	H	smed
Geranium dissectum L.	T	smed-subatl
Geranium molle L.	T/H	med-smed
Glechoma hederacea L.	H/Ch	euroas
Gypsophila muralis L.	T	euroas
Heliotropium europaeum L.	T	med-smed
Hibiscus trionum L.	T	ist.smed
Holcus lanatus L.	H	subatl-smed
Hypericum perforatum L.	H	euroas.suboc-smed
Inula britannica L.	H	euroas.cont-smed
Kickxia elatine (L.) Dum.	T	smed
Kickxia spuria (L.) Dum.	T	smed
Lactuca saligna L.	T/H	med-smed
Lactuca serriola L.	H/T	smed
Lamium purpureum L.	T	euroas.smed
Lathyrus tuberosus L.	G	euroas.cont
Leontodon autumnalis L.	H	bor-subatl
Lepidium draba L.	H	med
Leucanthemum vulgare Lam.	H	ist.smed
Linaria vulgaris Mill.	H	euroas
Lotus corniculatus L.	H	euroas.suboc-smed
Malva sylvestris L.	H	cosm
Marrubium vulgare L.	H	med-smed-euroas.cont
Medicago lupulina L.	T/H	euroas-smed
Mentha arvensis L.	H	bor-euroas
Mentha longifolia (L.) Huds.	H	smed-euroas
Muscari racemosum (L.) Mill.	G	smed
Myosotis arvensis (L.) Hill.	H/T	bor-euroas
Ornitogalum umbellatum L.	G	subatl-smed
Oxalis stricta L.	H	adv
Papaver rhoeas L.	T	euroas-med
Petrorhagia saxifraga (L.) Link.	H	smed
Phleum pratense L.	H	cosm
Picris echioides L.	T	med
Plantago lanceolata L.	H	euroas.suboc
Plantago major L.	H	cosm
Plantago media L.	H	euroas
Poa annua L.	T	cosm
Poa trivialis L.	H	cosm

1	2	3
Polygonum aviculare L.	T	cosm
Polygonum lapathifolium L.	T	euroas.suboc
Portulaca oleracea L.	T	adv
Potentilla reptans L.	H	cosm
Prunella vulgaris L.	H	bor-euroas
Pteridium aquilinum (L.) K. in D.	G	cosm
*Ranunculus arvens*is L.	T	med-euroas
Ranunculus repens L.	H	bor-euroas
Raphanus raphanistrum L.	T	med-smed
Reseda lutea L.	H/T	smed-med
Rorippa sylvestris (L.) Bess.	H	euroas.suboc-smed
Rosa canina L.	NP	euroas.suboc-smed
Rubus caesius L.	NP	euroas-smed
Rubus ulmifolius Schott.	NP	smed-med
Rumex acetosa L.	H	circ
Rumex crispus L.	H	cosm
Rumex obtusifolius L.	H	mod.cont-subatl-smed
Sambucus ebulus L.	G/H	smed
Satureja hortensis L.	T	ist.med
Satureja montana L.	T	med
Scrophularia nodosa L.	H	euroas.suboc
Senecio vulgaris L.	T	cosm
Setaria glauca (L.) Beauv.	T	cosm
Setaria viridis (L.) Beauv.	T	cosm
Solanum nigrum L.	T	cosm
Sonchus oleraceus L.	T/H	cosm
*Sorghum halepens*e (L.) Pers.	G	adv
Spergula arvensis L.	T	cosm
Stachys palustris L.	H	euroas
Stellaria media (L.) Vill.	T	cosm
Tanacetum vulgare L.	H	euroas.suboc
Taraxacum officinale Weber	H	cosm
Thlaspi arvense L.	T	euroas-smed
Tribulus terrestris L.	T	cosm
Trifolium pratense L.	H	euroasian. subocean
Trifolium repens L.	H	cosm
Urtica dioica L.	H	bor-euroas
Verbena officinalis L.	H	cosm
Veronica chamaedrys L.	H	circ
Veronica persica Poir.	T	cosm
Vicia cracca L.	H/SH	bor-euroas
Vicia sativa L.	T/ST	cosm
Viola arvensis Murr.	T/H	euroas-suboc
Xanthium italicum Moretti	T	adv

[1] Life forms: G-Geophytes, H-Hemikryptophytes, Ch-Chamaephytes, T-Terophytes, NP-Nanophanerophytes, S-Scandentophytes.
[2] Floral elements: adv-adventive, bor-Boreal, euroas-Eurasian, smed-sub-Mediterranean, suboc-subocean, subatl-subatlantic, circ-circumpolar, cont-continental, med-Mediterranean, ist-Eastern, cosm-cosmopolitan, atl-Atlantic, mod.cont- moderately continental.

During the two-year study of weed flora in vineyards of B&H it was determine 133 species of vascular plants covered with 112 genera, 39 families, 4 class and 2 sections.

The analysis of the biological spectrum showed the existence of five life forms and we can say that studied flora has therophytic-hemikryptohpytic-geophytic character. In the biological spectrum of weed flora has been determine dominance of therophytic species (T) to which belongs 61 representative, or 45.86% of the total number of species.

Analysis of floristic elements has shown a 84 different floristic elements that classified into 9 floristic groups. Chorological analysis indicates dominance of Cosmopolitan, Eurasian, Mediterranean, Boreal, Adventive and sub-Mediterranean floral elements that comprise 125 species, or 93.99% of total number of the identified species. Phytogeographical analysis has been determined dominance of Cosmopolitan (26.32%), Eurasian (20.30%) and Mediterranean (13.53%) floral elements.

DISCUSSION

Taxonomic structure distribution is similar to taxonomic structure of weed flora of vineyards in Serbia (Šinžar and Živanović, 1992; Crnčević et al., 1992; Živanović, 1988) and the vineyards in Croatia (Dujmović-Purgar and Hulina, 2004; Vrbek, 2000).

Significant richness of weed flora is a consequence the existence of two regions with different environmental conditions, different intensity of anthropogenic influences, traditions and systems of grape vine growing.

The result is, a complex and diverse composition of weed flora and significant floristic differences in the continental and sub-Mediterranean winegrowing region of B&H. Also, it is important a presence of Cosmopolitan and Adventitious species which are intensively spread, and beyond typical weed and weed-ruderal species within the flora of vineyards B&H it is appears a significant number of ruderal and grassland species.

Weed flora of vineyards in the B&H has great floristic abudance because existence of continental and sub-Mediterranean wine-growing regions with different climatic, pedological, orographic and floristic characteristics. Studied flora has larger number of species than flora of vineyards in the region of Fruška Gora (Šinžar and Živanović, 1992) that consisting 74 weed species and weed flora of vineyards north-western Croatia

(Dujmović-Purgar and Hulina, 2004) represented by 109 species. In Malayer city in more than 10.000 ha under grape cultivation Rostami and Ahmadi (2014) were identificated only 51 species from 22 families, which indicates poorer weed flora.

According to the floristic richness weed flora in vineyards of B&H is, a much poorer than the weed flora of orchards of B&H (Kojić et al., 2005) in which it was determine 226 weed species from 45 families, and much richer than weed flora of row crops and small grains Pannonian Basin Republic of Srpska (Šumatić, 1997) in which it was represented 85 weed species from 28 families.

Regarding the dominance of terophytes, the biological spectrum is similar to the biological spectrum of weed flora in vineyards near Belgrade (Živanović, 1988) where terophytes are represented with 56.6%. Dujmović-Purgar and Hulina (2004) in the vineyards of northwestern Croatia have noted dominance of hemikryptophytes (51.38%) and terophytes (34.86%).

In the areal spectrum of weed flora in vineyards of B&H, dominat are species of wide distribution, which is characteristic of anthropogenic habitat and usual for weed flora. Territory of B&H, in plant-geographical terms, belongs to euro-Siberian - north American and Mediterranean region. These regions are differentiated in the provinces of: illyrian, moesian and centraleuropean. It is, certainly, a consequence of the presence of 84 different floral elements in areal spectrum of weed flora vineyards of B&H, in which dominate are Cosmopolitan and Eurasian group of floral elements, that make almost a half of the total number, and significant participation has Mediterranean, Boreal, Adventive and sub-Mediterranean group of floral elements. Kojić and Pejčinović (1982) reported a similar range of areal types of weed flora of Kosovo, with special emphasis of the representation sub-Mediterranean floristic elements (9.10%). Šumatić (1997) analysis of the areal spectrum of weed flora of row crops and small grains Pannonian Basin of the Republic of Srpska observed a significant participation of sub-Mediterranean floristic elements as a result of impact of sub-Mediterranean climate. Kojić et al. (2005) in the weed flora of orchards in B&H determine a great diversity of floral elements, dominance floral elements of wide distribution and greater percentage of the sub-Mediterranean floral elements (14.60%).

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THE INFLUENCE OF TEMPERATURE ON GERMINATION OF RAGWEED (*Ambrosia artemisiifolia* L.), WILD OAT (*Avena fatua* L.), COMMON COCKLEBUR (*Xanthium strumarium* L.) AND WEEDY SUNFLOWER (*Helianthus annuus* L.)

Markola Saulić, Darko Stojićević, Dragana Božić and Sava Vrbničanin*
University of Belgrade, Faculty of Agriculture, Belgrade – Zemun, Serbia
**sava@agrif.bg.ac.rs*

ABSTRACT

In the laboratory conditions, the effect of temperature on germination of four invasive species in the territory of the Republic of Serbia is observed: wild oat (*Avena fatua* L.), ragweed (*Ambrosia artemisiifolia* L.), common cocklebur (*Xanthium strumarium* L.) and weedy sunflower (*Helianthus annuus* L.). Seeds were germinated seven days in the dark and at various temperatures from 10-35°C. Based on the established percentage of germination rate and germination, it was concluded that temperatures had different effects on seed germination.

Key words: temperature, seed germination

INTRODUCTION

Seed germination depends on the environmental conditions and the characteristics of the seeds, as well as their interactions. Awareness of the germination of certain plant species is very useful for understanding the potential and invasiveness of those weeds. Each plant species for germination requires specific environmental conditions, including soil moisture, temperature, oxygen availability, presence/absence of light, microbial activity and nitrate content in soil (Baskin and Baskin, 1990). Some authors believed that temperature is a major factor for the germination process (Forcella, 1998).

Wild oat (*Avena fatua* L.) is a species of early spring that germinate and sprout at a minimum temperature of 1-2˚C (with optimum temperature for germination 16-20˚C) (Vrbničanin and Šinžar, 2003). For this species very strong dormancy is typical and it was used as a model plant for reseracing dormancy (Foley, 1992). Common cocklebur (*Xanthium strumarium* L.) is one of the most common, competing weeds found in crops across Serbia (Vrbničanin et al., 2009). Common cocklebur seed is contained in burs. Lower seed isn't dormant, while the top seed is dormant and it doesn't germinate for months to a year after maturity (Barton, 1962). It was late spring species whose seeds germinate and sprout at the optimum average daily temperature 14-16°C (Vrbničanin and Šinžar, 2003). Growing weedy sunflower (*Helianthus annuus* L.) is also late spring species and it can be in the soil for many years to maintain germination (Vischi et al. 2006). Ragweed (*Ambrosia artemisiifolia* L.) belongs to the spring species and seeds germinate at temperatures ranging from 6 to 32°C, and the optimum is achieved at 20-22°C (Vrbničanin and Šinžar, 2003), while illumination increases germination (Ristić et al., 2008). It was confirmed that ragweed seeds can germinate in conditions that are unfavorable for some other species, such as soil salinity (DiTommaso, 2004). Seeds of ragweed cannot germinate immediately after the decline of the mother plant, due to very strong primary dormancy (Baskin and Baskin, 1980). Germination occurs after stratification during winter. The seeds that did not germinate in the spring, enter the secondary dormancy and cannot germinate until

they live again a phase of stratifaction next winter (Milanova and Nakova, 2002). It has been shown that in laboratory conditions seed germination was higher due to stratification at -8°C compared to +4 °C (Konstatinović et al., 2013).

Despite numerous studies, the germination of weed seeds, as well as the influence of various biotic and abiotic factors on the complexity of the process of germination, there is a need for such research. The aim of this study was to evaluate the germination of four weed species (wild oat, ragweed, weedy sunflower and common cocklebur). Knowing the optimum temperature range in which a specific weed species germinates could shed light on the biology of such a species and can be useful in predicting significant flushes of emergence, leading to more proactive and practicable control measures.

MATERIAL AND METHODS

For the purposes of this research, seeds of wild oat, ragweed, common cocklebur and weedy sunflower were collected from different localities in Serbia. Namely, seeds of wild oat collected at the locality Radmilovac, seeds of ragweed at the village Čestereg, and seeds of common cocklebur and weedy sunflower at the locality Surčin. The plants were removed at the stage of physical maturity of seeds. From collecting separated seeds to starting the experiment, they are stored at room temperature (20-25°C) about six months. In 9 cm diametar Petri dishes with filter paper 5 burn of common cocklebur and 20 weedy sunflower seeds, wild oat and ragweed were placed, and then it was added 5 ml of distilled water. The germination was studied in an aincubator (Vinder CE and Memmert) in the dark at the following temperatures: 10, 15, 20, 25, 30, 35°C

Germination was monitored every day during seven days. Each treatment consisted of 2 sets of 8 replications for all species.

The data were analyzed and calculated the percentage of seed germination and the germination rate by the following formula:

$$M= n_1/t_1 + n_2/t_2 ...+n_x/ t_x,$$

where M is germination rate and n_1, n_2 ...n_x stand for the number of seeds that germinated on days t_1, t_2 ... t_x starting from the beginning of imbibition (Maguire, 1962).

All data was analyzed by one-way ANOVA (F-values) using statistical software Statistica 5.0. Differences between populations were tested using t-test.

RESULTS

After seven days of seeds imbibition in the dark at different temperature regimes, generally the best germination had seed of wild oat. Even 90% of the seeds of this species germinated, while maximum germination of ragweed seeds was actually only 30%. The seeds of common cocklebur germinated with 43.33% and 40% of sunflower (Table 1).

Seeds of wild oat has the best germination at a temperature of 10°C (90%) and the lowest at 35°C (6.87%). Weedy sunflower seeds and common cocklebur have mutually similar results, ie. it turns out that the optimum temperature for germination of both types was 25°C. At this temperature, weedy sunflower showed 40% germination and common cocklebur of 43.33%. However temperature of 10°C is not at all conducive to germination of common cocklebur because no seeds germinated. To weedy sunflower the temperature of 30°C was at least corresponded, only 5% of the seeds has germinated. The highest percentage of seed germination of ragweed was recorded at a temperature of 25°C (30%), while at a temperature of 10°C not a single seed germinated

Analysis (t-test) data on % of germination of wild oats and ragweed at different temperatures showed statistically significant differences ($P<0.01$) in germination between different temperatures in most cases, while the weedy sunflower had no statistically significant difference between the following treatments: 10 and 15°C; 10 and 20°C; 15 and 20°C; 15 and 25°C; 20 and 25 and 30°C and 35°C. Differences in germination between the temperatures of common cocklebur in most cases were not statistically significant ($P>0.05$) (Table 2).

Based on the daily readings of examined germination of weed species germination rates were calculated to indicate the dynamics of seed germination. The germination rate of wild oat seed was highest at 25°C (7.90 seeds/day) and the lowest at a temperature of 30°C (0.79 seeds/day). At a temperature of 10°C at common cocklebur seed, was noted the rate of germination 0, while the highest (3.85 seeds/day) was at a temperature of 25°C. In the weedy sunflowers highest germination was at a temperature of 20°C (2.43 seeds/day) and the lowest at 25°C (0.08 seeds/day. In the case of the highest rate of germination of ragweed (7.84 seeds/day) is achieved at a temperature of 30°C, while at 10°C (0 seeds/day) the rate was the lowest (Table 3). Seeds of ragweed that were exposed to the treatment of light/dark 16h/8h and temperature of 24°C had the highest rate of germination (3.36 seeds/day),

Table 1. Percentage of germinated seeds of wild oat, common cocklebur, weedy sunflower and ragweed at different temperatures on the seven day of the experiment

Temperature (°C)	Percent of germination			
	Wild oat	Common cocklebur	Weedy sunflower	Ragweed
10	90.00±8.94	0.00±0.00	30.0±8.16	0.00±0.00
15	85.62±13.15	36.67±5.77	32.5±9.57	13.54±5.37
20	73.75±14.55	36.51±23.09	35.0±1.0	26.87±9.46
25	70.62±18.06	43.33±5.77	40.0±0.0	30.0±6.44
30	8.75±2.04	36.67±23.09	5.0±0.82	23.33±6.21
35	6.87±7.93	36.67±23.09	5.0±1.0	18.12±6.43

Table 2. Statistically significant differences in germination (%) in wild oat, common cocklebur, weedy sunflower and ragweed at different temperatures (t-test)

T(°C)	10	15	20	25	30	10	15	20	25	30
	Wild oat					Common cocklebur				
15	ns					**				
20	**	*				**	ns			
25	**	*	ns			**	**	ns		
30	**	**	**	**		**	ns	ns	ns	
35	**	**	**	**	ns	**	ns	ns	ns	ns
	Weedy sunflower					Ragweed				
15	ns					**				
20	ns	ns				**	**			
25	*	ns	ns			**	**	ns		
30	**	**	**	**		**	**	ns	**	
35	**	**	**	**	ns	**	*	**	**	*

p<0,001**, 0.01<p<0.05*, p>0,05 ns-differences are not statistically significant

Table 3. The influence of different temperatures on germination rate of wild oat, common cocklebur, weedy sunflower and ragweed

Temperature (°C)	Germination rate			
	Wild oat	Common cocklebur	Weedy sunflower	Ragweed
10	5.66±1.72	0.00±0.00	0.59±0.22	0.00±0.00
15	7.75±1.46	2.70±0.30	1.73±0.91	1.15±0.47
20	7.88±1.68	2.15±0.15	2.43±0.74	5.12±2.26
25	7.90±2.00	3.85±0.22	0.08±0.15	6.67±1.57
30	0.79±0.19	3.54±0.11	0.67±0.2	7.84±2.21
35	1.12±0.30	2.90±0.30	0.79±0.1	5.86±1.92

while the lowest germination had the seed that was sprouted in darkness and at constant temperature of 22°C (1.11 seeds/day). The results show that for all species except for weedy sunflower, in most cases, there is a significant difference (p<0.01) in germination rate at various temperatures (Table 4).

Table 4. The significance of differences in the rate of germination of wild oat, common cocklebur, weedy sunflower and ragweed at different temperatures (t-test).

T(°C)	10	15	20	25	30	10	15	20	25	30
	Wild oat					Common cocklebur				
15	**					**				
20	**	ns				**	**			
25	**	ns	ns			**	**	**		
30	**	**	**	**		**	**	**	**	
35	**	**	**	**	ns	**	ns	**	**	**
	Weedy sunflower					Ragweed				
15	ns					**				
20	**	ns				**	**			
25	ns	*	**			**	**	*		
30	ns	ns	ns	ns		**	**	**	ns	
35	ns	ns	ns	ns	ns	**	**	ns	ns	*

p<0,001**,0.01<p<0.05* p>0,05 ns-differences are not statistically significant

DISSCUSION

The results indicate that wild oat seed germinates better at lower and ragweed, weedy sunflower and common cocklebur seeds at higher temperatures, which was expected given that these three species belong to the group of late spring, and a group of wild oats of early spring weeds.

Knowing the optimum temperature for germination of oats as weeds in small grains is of great importance because of better forecast time for germination and sprout. Stougaard and Xue (2004) have confirmed that the reduction in wheat yield due to competition with wild oat of moving from 47 to 58%. The our study confirmed that oats germinate best at a low temperature of 10°C and the lowest seed germination had a temperature of 35°C. The effect of temperature on the process of seed germination of *A. fatua* and *A. ludoviciana*, investigated by Fernandez-Quinantila et al. (1990), where they got an opposite observation that the germination of seeds of *A. fatua* at temperatures lower than 10°C is worse, and at temperatures above 20°C better. Seeds of common cocklebur showed the highest % germination and germination rate at 25°C. Norsworthy & Oliveira (2007) concluded that the optimal temperature in field trials for seed germination of common cocklebur is 35 and 40°C. Based on the daily readings of seed germination of weedy sunflower, germination rates were calculated to indicate the dynamics of seed germination. The results showed that the germination rate is not in accordance with established % of seed germination. Namely, at a temperature of 25°C, the highest % of germination was recorded, and also at the same temperature, the lowest rate of germination. Also Jovičić et al. (2011) concluded that the optimum temperature for germination of weedy sunflower is 25-30°C.

Ragweed was germinated in a smaller percentage of seeds at most favored temperature of 25°C. One of the possible reasons for poor germination ragweed is that it is characterized by a very strong seed dormancy (Williemsen and Rice, 1972), but also that the seed was sprouted in the dark. In the following experiments to determine the seeds of this species germinate better in the light of which coincided with the results of Ristic et al. (2008).

Our study agrees with the observations of Sermons et al. (2008), that the temperature effects on seed germination. The results indicate the existence of significant statistical difference between germination at different temperatures. Only the results at % of germination of common cocklebur and weedy sunflower germination rate was not statistically significant. These results are important, because a better understanding of the biological properties of the seed (of viability, germination, periodic germination and dormancy) can be useful for predicting the spread of weeds, their invasiveness and the development of more effective strategies to control weeds in arable and on non-agricultural areas.

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CARDINAL TEMPERATURES AND DYNAMICS OF GERMINATION OF COMMON RAGWEED (*Ambrosia artemisiifolia* L.) SEEDS COLLECTED IN ZEMUN

Vladan Jovanović[1*], Jelena Juzbašić[2], Ivana Dragićević[3], Vaskrsija Janjić[1], Bogdan Nikolić[4] and Danijela Mišić[5]

[1]Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Serbia
[2]M.Sc. graduate of the University of Belgrade-Faculty of Biology, Studentski trg 16, 11000 Belgrade, Serbia
[3]University of Belgrade-Faculty of Biology, Studentski trg 16, 11000 Belgrade, Serbia
[4]Institute for Plant Protection and Environment, Teodora Drajzera 9, 11000 Belgrade, Serbia
[5]Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia
**e-mail: vladan.jovanovic@pesting.org.rs*

ABSTRACT

The reaction of seeds to various environmental stimuli is highly important as they strongly affect the process of germination and seedling ability to use reserves that are important for initial development stages and their full capacity as autotrophic organisms to utilize the energy of the sun.

Seeds of common ragweed (*Ambrosia artemisiifolia* L.) were sampled on the outskirts of Zemun (Altina suburb) and incubated (non-stratified) at nine constant temperatures in order to determine the cardinal germination temperatures. The following temperatures were tested: 3, 7, 11, 15, 19, 23, 28, 33 and 37 °C. Throughout the germination experiment, seeds were exposed to light only briefly during each counting session (3-5 min). Germinating seeds were counted at 12 h intervals during the first week, while counts were taken at 24 h intervals in the following week, and at 2-3 days intervals until the end of experiment. Distilled water was added to provide germination and to prevent seed drying. Germinating seeds were removed from petri dishes after counts. The experiment lasted six weeks.

No seeds were found to germinate at 3°C. The time required for germination was longest for seeds exposed to the two next lowest and the highest temperature (7, 11 and 37 °C), while plateau of germination was reached most rapidly by those exposed to 23, 28 and 33 °C. Seeds exposed to all temperatures reached their germination maximum 21 days after the beginning of experiment at the latest.

The percentage of germinating seeds was highest at 23 °C but the speed of germination was highest at 28 °C. The base temperature (T_b) was 3.4 °C, maximum temperature (T_m) for 50 % germinating seeds was 39.1 °C. The optimum temperature for germination was 31.5 °C.

INTRODUCTION

The reaction of seeds to various environmental stimuli is highly important as they strongly affect the process of germination and their ability to use reserves that are important for initial development stages of young plants and their full capacity as autotrophic organisms to utilize the energy of the sun (Nešković et al., 2010). Since 1860, three cardinal (main) points have been identified within the temperature range in which seeds of any particular species are able to germinate (Bewley and Black, 1994). Minimum or base temperature (T_b)

is the lowest temperature at which germination occurs; optimum temperature (T_o) is one at which germination is most rapid (there is usually a variably small range of such optimum temperatures, rather than a single value of T_o) and maximum temperature (T_m) as the highest temperature at which germination occures (Labouriau and Osborn, 1984; Orozco-Segovia et al., 1996).

Cardinal temperatures of any given species reflect its scope of adaptation to environmental conditions, which enables its seeds to germinate under the most favourable conditions for seedling growth and development (Bradford and Somasco, 1994). The degree of dormancy may significantly affect the temperature range between T_b and T_m (Alvarado and Bradford, 2002).

Base temperature is a crucial parameter for predicting a period in which seeds will be able to germinate. Knowing the base temperature is essential for explaining the effect of temperature on germination rate (GR) (Trudgill et al., 2005).

Seed properties of the common ragweed, a strong allergenic and highly harmful invasive weed species, have been studied extensively. Cardinal temperatures for germination of common ragweed seeds have been determined in several studies (Shrestha et al., 1999; Sartorato and Pignata, 2008; Gardarin et al., 2010; Guillemin et al., 2012) but not for samples from Serbia**.** The present study is focused on determining the dynamic of germination at constant temperatures and cardinal germination temperatures of ragweed seeds sampled in the environs of Zemun, Serbia.

MATERIALS AND METHODS

Seeds of common ragweed (*Ambrosia artemisiifolia* L.) were collected on the outskirts of Zemun (Altina suburb) and incubated (non-stratified) at nine constant temperatures in order to determine the cardinal germination temperatures. The following temperatures were tested: 3, 7, 11, 15, 19, 23, 28, 33 and 37 °C. Batches of 50 seeds were placed into 6 cm Petri dishes containing 2 ml of distilled water. Three Petri dishes were used to test each experimental treatment. Throughout the germination experiment, seeds were exposed to light only briefly during each counting session (3-5 min). Germinating seeds were counted at 12 h intervals during the first week, while the counts were taken at 24 h intervals in the following week, and 2-3 days intervals until the end of experiment. Distilled water was added to provide germination and to prevent seed drying. Germinating seeds were removed from petri dishes after counts. The experiment lasted six weeks.

A mathematical formula based on regression coefficient (Yang et al., 1995; Steinmaus et al, 2000) was used to calculate the base temperature (T_{base}):

$$T_{base} = \frac{\sum_{i=1}^{n} T_i \sum_{i=1}^{n} t_i T_i - n \sum_{i=1}^{n} t_i T_i^2}{\sum_{i=1}^{n} t_i \sum_{i=1}^{n} T_i - n \sum_{i=1}^{n} t_i T_i}$$

where T_i is germination temperature in each petri dish i; t_i is germination time required for 50% of the seeds to germinate in each petri dish i; n is the total number of petri dishes.

Maximum temperatures (T_m) were determined by regression analysis based on germination rates (1/t) per 50% of the seeds that germinated at supra-optimal temperatures.

Statistical analyses were performed using the STATGRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985-1989, USA). The percentage data were *arcsin* transformed before statistical analysis. The data were subjected to one-way analysis of variance (ANOVA). Differences between means were evaluated by Fisher's LSD test calculated at the confidence level of $P \leq 0.05$. The results of statistical analysis are marked with appropriate letters in the figures, and samples bearing the same letters have no significant statistical difference.

RESULTS AND DISCUSSION

No seeds were found to germinate at 3°C (Figure 1). The percentage of germinating seeds was highest at 23 °C. The time required for germination was longest for seeds exposed to the two next lowest and the highest temperature (7, 11 and 37 °C), while plateau of germination was reached most rapidly by those exposed to 23, 28 and 33 °C (Figure 2). Seeds exposed to all temperatures reached their germination plateau 21 days after the beginning of experiment at the latest.

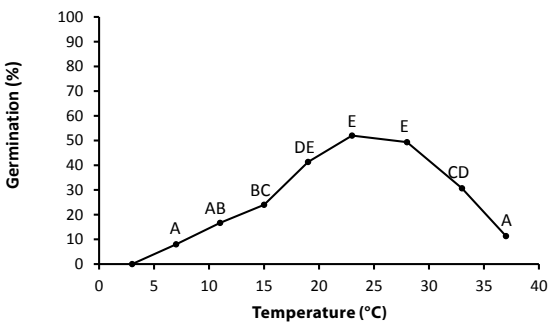

Figure 1. Germination of common ragweed seeds at constant temperatures.
Samples bearing the same letters have no significant statistical difference.

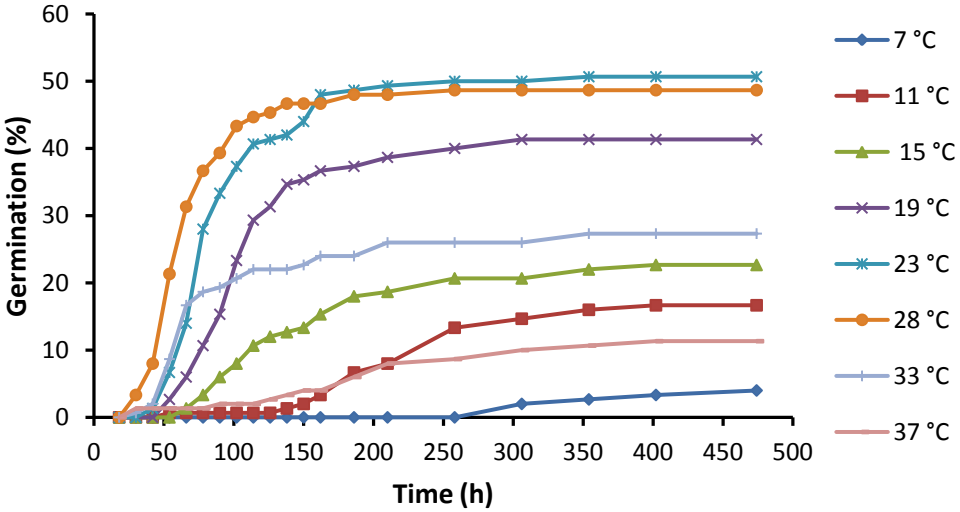

Figure 2. Dynamic of germination of common ragweed seeds at constant temperatures

A formula for calculating base temperature included data on all suboptimal temperatures, and the base temperature (T_b) for germination of common ragweed seeds was calculated as 3.4 °C. The formula used here had been shown in other studies to produce T_b values that do not diverge more than 1 °C from those calculated by regression analysis (Steinmaus et al., 2000).

The calculated T_b of 3.4 °C is close to the T_b values reported in literature for common ragweed seeds. Shrestha et al. (1999) found a T_b = 3.6 °C, based on probit analysis; Guillemin et al. (2012) chose regression analysis and detected a base temperature of 3.6° C, while Sartorato and Pignata (2008) also used regression analysis but their T_b was 3.4 °C.

Maximum temperature (T_m) in our study was determined by regression analysis based on germination rates (1/t) per selected germination percentage at supraoptimal temperatures. The T_m was 39.1 °C for 50% of germinated ragweed seeds (Figure 3). The optimum temperature for germination of 50% of the seeds was 31.5 °C.

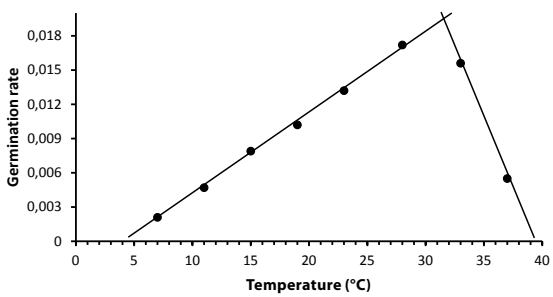

Figure 3. Germination rate for 50 % of the germinated seeds of common ragweed at different temperatures

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TOXICOLOGY AND ECOTOXICOLOGY

ТОКСИКОЛОГИЯ И ЭКОТОКСИКОЛОГИЯ

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CYTOGENETIC MONITORING IN A SERBIAN POPULATION EXPOSED TO PESTICIDES: USE OF MICRONUCLEI

Dubravka Jovičić[1], Ljiljana Radivojević[2] and Vaskrsija Janjić[2]

[1]Faculty of Applied Ecology „Singidunum" University, Belgrade, Serbia,
[2]Institute of Pesticides and Environmental, Belgrade, Serbia
dubravka.jovicic@futura.edu.rs

ABSTRACT

Aim of this study was to assess the damage to the genetic material, detected with the micronucleus test in workers occupationally exposed to pesticides. The research included 119 subjects divided into three groups: the control group, there were 39 subjects, in the group exposed to pesticides (producers) were 40 subjects and 40 subjects - applicants in the field, were analyzed in this paper. Mann-Whitney U test showed a statistically significant difference between the mean values of the parameters of all variables compared to the control group. Significant differences between males in production and application for parameters MN4, then among nonsmokers in the production and application for parameters MN2, MN3, MN4and NB, as well as in the whole population observed between control and applicants for parameter MN2, and between producers and applicants for parameter MN3. A statistically significant difference in relation to the cytogenetic parameters studied was registered between respondents who work in the production of pesticides and of those who work in the field. The results suggest that workers who are working in the field do not use adequate personal protective equipment. The continuous biological monitoring of workers exposed to pesticides is required.

Key words: pesticides, workers, micronucleus test, genetic damage

INTRODUCTION

Pesticides constitute a heterogeneous category of chemicals specifically designed for the control of pests. Their application is still the most effective and accepted means for the protection of plants from pests, and has significantly contributed to the enhanced agricultural productivity and crop yield (Bolognesi, 2003). In recent years the use of pesticides in agriculture has been increasing steadily. At present there are more than 1000 chemicals classified as pesticides (Torres et al., 1992). A total of about 890 active ingredients are registered as pesticides in USA and currently marketed in some 20 700 pesticide products (Tomlin, 2009).

Moreover, in the case of occupational contact with pesticides, there is great interindividual variability in the degree of exposure and it generally involves complex mixtures of many kinds of compounds. Exposure to pesticides has been associated with increases in the incidence of non-Hodgkin´s lymphoma (Hardell et al., 1999; Zheng et al., 2001), pancreatic, stomach, liver and bladder cancer (Shukla et al., 2001), Parkinson´s disease (Gauthier et al., 2001), immunotoxicity (Corsini et al., 2013) and undesirable reproductive outcomes (Arbukle et al., 2001; Mostafalou and Abdollahi, 2013) among others.

Populations occupationally exposed to pesticides, which are in direct contact almost daily, constitute one of the human groups at genotoxic risk. Many biomonitoring studies have evaluated cytogenetic effects in pesticide exposed workers from different countries (Pasastor et al., 2002). There are only a few reports of

health effects due to chronic occupational exposure to pesticides in developing countries. People in developing countries are at higher risk from chronic exposure to these chemicals because of poor working conditions and an unawareness of the potential hazards in production, trade and application (Baker et al., 1978).

Main aim of the present study is cytogenetic monitoring of Serbian population occupationally exposed to a comlex mixture of pesticides.

MATERIAL AND METHODS

The exposed group was composed of 40 (E1) individuals working in different units of pesticide production (pesticide formulation units for liquid and solids products) and 40 (E2) individuals working on the application of pesticides in the field. The study included a control group with 39 subjects. Control individuals were not occupationally exposed to any particular chemical agent.

Table 1 shows the characteristics of the studied groups regarding sex (male or female), age, duration of occupational exposure to pesticides (DOE in years), smoking habit (smoker or non – smoker). Genetic damage detected with the micronucleus test (CBMN – cytokinesis-blockmicronucleus) in workers occupationally exposed to pesticides.

Standard protocol for the classical cytogenetic analysis was used in the CBMN test (IAEA, 2001). The slides were analyzed by light microscopy (Olympus BX-51, magnification 1000x). Binuclear cells are analized according to a standard protocol and the prescribed criteria of the projekt HUMN (Human MicroNucleus) international collaborative study (Bonassi et al., 2001). One thousand binucleate cells per subjects and per dose were analyzed and the total number of MN found and their distribution recorded. The parameters that describe the distribution of micronuclei are defined as number of binuclear cells with 1-4 nuclei.

The following inclusion criteria were used to score the micronuclei, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in peripheral blood samples.

In addition to descriptive statistics, appropriate non–parametric statistical methods were used: Mann–Whitney U-test, Pearson's X^2 test and Spearman rank test (non-parametric correlation test) on the whole sample (control-exposed) and the exposed group only. The softwares used for data analyses were STATISTICA (StatSoft, Tulsa, OK) and SPSS version 10.0 (SPSS Inc., Chicago, IL).

RESULTS

Here we report the results of cytogenetic monitoring of 80 occupationally exposed workers and 39 matched controls using the cytokinesis-block micronucleus (CBMN). Table 1 give the main characteristics of the population studied.

Table 1. Characteristics of the population studied

Unexposed - Control					
			Aged	Smoking habit	
	n	Mean±SD	Median; range	Smokers	Non-smokers
Men	20	36.70±7.64	(37; 23-50)	10	10
Women	19	41.16±7.25	(43; 26-56)	4	15
Producers					
			Aged	Smoking habit	
	n	Mean±SD	Median; range	Smokers	Non-smokers
Men	24	40.46±8.02	(41.0; 25-54)	10	14
Women	16	40.44±6.94	(40.5; 30-58)	8	8
Applicants					
			Aged	Smoking habit	
	n	Mean±SD	Median; range	Smokers	Non-smokers
Men	19	38.53±7.32	(37; 27-53)	12	7
Women	21	37.48±8.57	(38; 22-52)	15	6
∑	119			59	60

The Mann-Whitney U-test indicated statistically significant differences of average values examined variables in comparison to the control group. The results of MN-tests for entire population (exposed and control) are shown in the Table 2. On the level of control group of subjects an established average (8.44±3.35) MN (median 8 MN) while the span of individual values varied from 3 to 18 MN per 1000 binuclear cells. Workers employed in pesticide production (E1) had an average (14.55±5.48) MN (median 14.5) while the span of individual values for these subjects was from 4 to 29 MN, and field workers (E2) had an average of (16.31±4.79) MN (median 16) while the span of individual values was from 8 to 31.

Table 2. Results of MN-test for studied population

Unexposed - Control			Producers		Applicants	
Paramtear	Mean±SD	Median; range	Mean±SD	Median; range	Mean±SD	Median; range
Studied population						
MN	8.44±3.35	8; 3 - 18	14.55±5.48	14.5; 4 - 29	16.31±4.79	16; 8 - 31
MN1	7.23±2.66	7; 3 - 14	10.58±3.13	10; 4 - 18	12.13±2.81	12; 4 - 18
MN2	0.56±0.75	0; 0 - 2 **a***	1.28±1.15	1; 0 - 6	1.10±1.03	1; 0 - 5 **b**
MN3	0.03±0.16	0; 0 - 1	0.43±0.21	0; 0 - 3 **a**	0.45±0.75	0; 0 - 3 **b**
MN4	0.00±0.00	0; 0 - 0	0.05±0.02	0; 0 - 1	0.20±0.46	0; 0 - 2
NB	0.51±0.97	0; 0 - 4	2.68±1.95	3; 0 - 7	2.58±1.30	3; 0 – 5
NPB	0.08±0.27	0; 0 - 1	0.48±0.32	0; 0 - 3	0.53±0.82	0; 0 - 3
Women						
MN	9.21±3.36	9; 4 - 18	14.50±5.62	16; 4 - 26	16.90±4.57	16; 10 - 26
MN1	7.63±2.75	8; 4 - 14	10.88±3.36	10.5; 4 - 18	13.14±2.63	12; 9 - 18
MN2	0.79±0.85	1; 0 - 2	1.00±1.26	0.5; 0 - 4	1.00±0.89	1; 0 - 2
MN3	0.00±0.00	0; 0 - 0	0.50±0.45	0; 0 - 2	0.33±0.58	0; 0 – 2
MN4	0.00±0.00	0; 0 - 0	0.06±0.05	0; 0 - 0	0.19±0.40	0; 0 -1
NB	0.58±1.07	0; 0 - 4	2.25±1.81	2; 0 - 7	2.71±1.23	3; 0 - 5
NPB	0.16±0.37	0; 0 - 1	0.38±0.21	0; 0 - 3	0.62±0.97	0; 0 - 3
Men						
MN	7.70±3.25	8; 3 - 13	14.58±5.50	14; 5 - 29	15.74±4.96	15; 8 - 31
MN1	6.85±2.57	7; 3 - 11	10.38±3.02	10; 5 - 17	11.00±2.62	11; 4 - 16
MN2	0.35±0.59	0; 0 - 2	1.46±1.26	1; 0 - 6	1.21±1.18	1; 0 - 5
MN3	0.05±0.22	0; 0 - 1	0.37±0.21	0; 0 - 3	0.53±0.40	0; 0 - 3
MN4	0.00±0.00	0; 0 - 0	0.04±0.02	0; 0 - 1 **a**	0.21±0.53	0; 0 - 2 **b**
NB	0.45±0.88	0; 0 - 3	0.04±0.02	0; 0 - 1	0.21±0.53	0; 0 - 2
NPB	0.00±0.00	0; 0 - 0	2.96±2.03	3; 0 - 7	2.42±1.39	3; 0 - 5
Smokers						
MN	9.21±3.91	8.5; 3 -18	15.33±5.52	14; 4 - 28	16.52±5.37	15; 8 - 31
MN1	7.86±3.06	8; 3 - 14	10.89±3.53	10.5; 4 - 18	11.70±2.85	12; 4 - 18
MN2	0.57±0.76	0; 0 - 2	1.39±1.29	1; 0 - 4	1.15±1.17	1; 0 - 5
MN3	0.07±0.27	0; 0 - 1	0.56±0.92	0; 0 - 3	0.52±0.85	0; 0 - 3
MN4	0.00±0.00	0; 0 - 0	0.00±0.00	0; 0 - 0	0.26±0.53	0; 0 - 2
NB	0.50±0.76	0; 0 - 2	2.78±2.29	2; 0 - 7	2.54±1.39	2.5; 0 - 5
NPB	0.00±0.00	0; 0 - 0)	0.34±0.26	0; 0 - 3	0.52±0.89	0; 0 - 3
Non-smokers						
MN	8.00±2.99	8; 3 - 13	13.91±5.49	15; 5 - 29	13.91±5.49	15; 5 - 29
MN1	6.88±2.40	6; 3 - 11	10.32±2.82	10; 5 - 15	10.32±2.82	10; 5 - 15
MN2	0.56±0.77	0; 0 - 2	1.18±1.59	0.5; 0 - 6 **a**	1.18±1.59	0.5; 0 - 6 **b**
MN3	0.00±0.00	0; 0 - 0	0.32±0.48	0; 0 - 1 **a**	0.32±0.48	0; 0 - 1 **b**
MN4	0.00±0.00	0; 0 - 0	0.09±0.03	0; 0 - 1 **a**	0.09±0.29	0; 0 - 1 **b**
NB	0.52±1.08	0; 0 - 4	2.59±1.68	3; 0 -6 **a**	2.59±1.68	3; 0 - 6 **b**
NPB	0.12±0.33	0; 0 -1	0.59±0.85	0; 0 - 1	0.60±0.85	0; 0 - 3

*- Mann-Whithey U-test; a, b - significant differences ($p<0.05$)

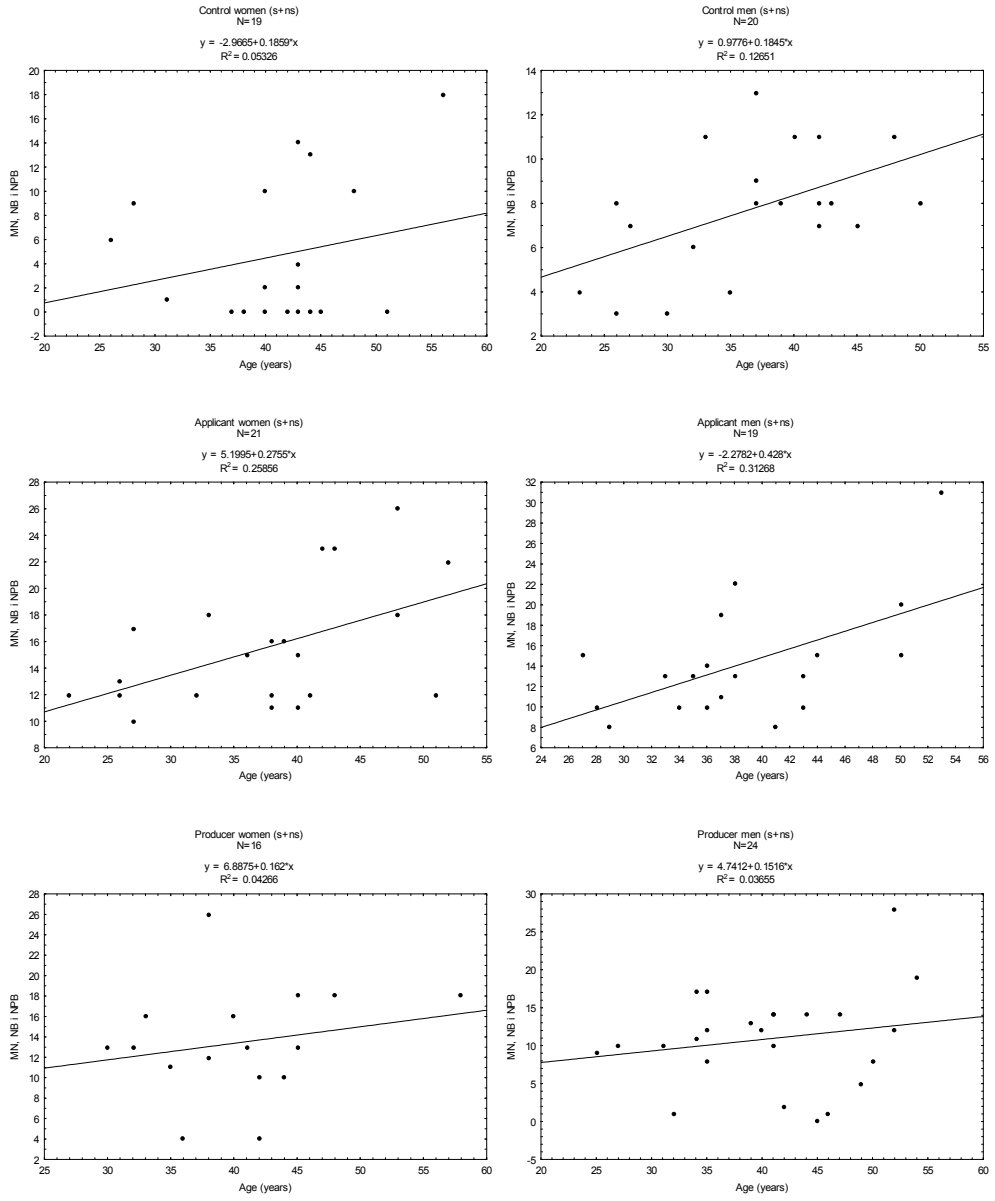

Figure 1. Correlations between MN, NB, NPB and age for men and women (smokers and non-smokers)

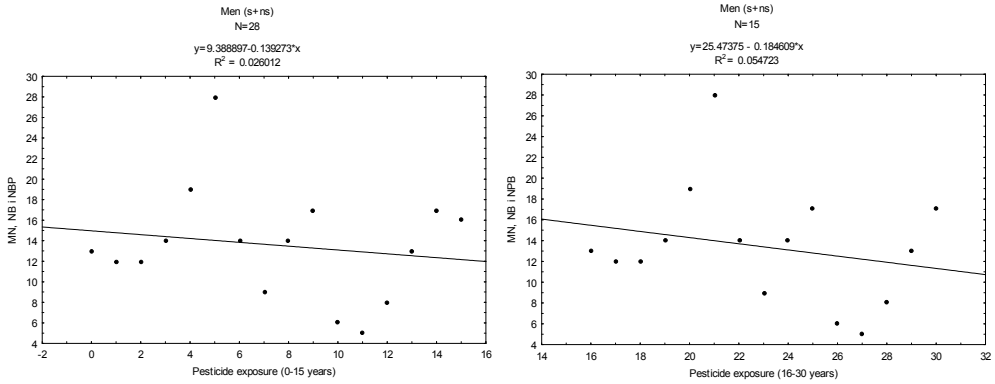

Figure 2. Correlations between MN, NB, NPB and pesticide exposure for men and women (smokers and non-smokers)

The performed correlation analysis determined statistically positive correlation between age and the total number of MN, NB and NPB for women (smokers and non-smokers) in control (R=0.23078), as well as women applicants (R=0.50850), i.e. women in production (R=0.20656). Also, statistically positive correlation was noticed between age and the total number of MN, NB and NPB for men (smokers and non-smokers) in control (R= 0.50200), as well as men applicants (R=0.55918) and men in production (R=0.19119) (Figure 1).

Figure 2 shows that correlation analysis determined statistically positive correlation between exposed work period of 0-15 years (R=0.16128), i.e. slightly higher for exposed work period of 16-30 years (R=0.23393) from the total number of MN, NB and NPB for men (smokers and non-smokers) in production and applicants.

DISCUSSION

The analysis of our results has shown that there is a statistically significant difference between control and exposed group in terms of examined parameters. The analyzed parameters relative to MN were analyzed in terms of sex, age and the smoking habits as factors that, according to the data from literature, can influence their basal incidence (Hagmar et al., 1994).

The analysis of our results has shown that the average values of the parameters that refer to genetic damages detected with MN test is higher in exposed groups of subjects than on the level of the control group where the determined average was (8.44±3.35) MN (median 8 MN) while for the employees in pesticide production (E1) it was (14.55±5.48) MN (median 14.5), and for field workers (E2) the average was (16.31±4.79) MN (median 16).

The span on individual values for this group is from 8 to 31 and it was the highest in comparison to both observed groups. The subjects with the highest number of MN are employed in pesticide application, that many studies (Bolognesi, 2003) distinguish as a point of the highest exposure. Besides that, blood for cytogenetic analyses was taken in the moment of the most intensive application when, in accordance with the data from literature, the highest incidence of genetic damage is expected (Bhalli et al., 2006). In our work, we noticed increased frequency of MN occurrence in exposed workers (men and women) compared to the control group. Presented data is in accordance with other studies which researched similar problems (Costa et al., 2006).

The analysis of our results in comparison with the age category was based in the literature data, that explain increased incidence of genetic damage with age with the combination of factors that include: a) cumulative effect of acquired mutation of genes involved in reparation DNA molecules, separation of chromosome and control point of cell cycle and b) numerical and structural aberrations of chromosome caused by exposure of endogenous toxins, inadequate diet and other mutagenic factors of the environment (Fenech et al., 2011; Jovičić et al., 2013).

All results of our study indicate that the level of cytogenetic damage was significantly affected by the pesticide exposure of subjects. They also indicate the need of permanent biomonitoring of persons occupationally exposed to various mixtures of pesticides.

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NICOSULFURON RESIDUES IN AGRICULTURAL SOIL

Sanja Lazić[1], Dragana Šunjka[1] and Nada Grahovac[2]

[1]University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, Novi Sad, Serbia
[2]Institute of field and vegetable crops, Maksima Gorkog 30, Novi Sad
draganas@polj.uns.ac.rs

ABSTRACT

Nicosulfuron is one of the most applicable sulfonylurea herbicides for control of annual and perennial weeds in maize. Its residues can persist in soil at phytotoxic concentrations and affect sensitive succeeding crops more than one season after treatment. Therefore, monitoring the trace levels of this herbicide in soil is a challenging task and demands highly efficient, selective, and sensitive analytical technique. This study was conducted for the purpose of investigating nicosulfuron residues presence in soil. Soil samples were collected before crop seeding, from two soil layers, surface (0-30 cm) and sub-surface (30-60 cm). Nicosulfuron determination and quantification were performed by HPLC with diode-array detection, using isocratic elution of mobile phase. Nicosulfuron was extracted from the soil sample with mixture of phosphate buffer (pH 7.4)/methanol (80/20, v/v) solution with clean-up on C18 extraction cartridge. The linearity of detector response showed that the calibration curves were linear with correlation coefficient (R^2) of 0.999. Relative standard deviations (*RSD)* of the retention times and of the peak areas were 0.79 and 1.06%, respectively and fulfilled the criteria of chromatographic measurements. The accuracy of the defined method was confirmed by the good results of recovery assay, while achieved limit of detection (LOD) and limit of quantification (LOQ) for nicosulfuron in soil were in accordance with SANCO/825/00 (SANCO, 2010). The proposed analytical procedure was applied for monitoring of nicosulfuron herbicide in soil. Average values of nicosulfuron residues in soil samples from surface and sub-surface horizon were 0.05 mg/kg.

Key words: nicosulfuron, residue, soil

INTRODUCTION

Modern agriculture depends to a large extent on herbicides used for control of weeds that compete with the crops. Herbicides represent about 50% of the demand for agricultural chemicals and are applied directly or indirectly to increase crop yields. Furthermore, herbicides protect crops from undue competition from weeds and enhance food nutrition quality of food. Their prolonged use involves the risk of their retention and accumulation in the environment, so the analysis of these compounds has become an important part of the monitoring program.

Stability of herbicides in the soil is also dependent on degradation manners. A very important indicator, which defines potential persistence of the herbicide active ingredient in the soil, is the half-life period (DT_{50}). The value of DT_{50} is a characteristic feature of individual active ingredients of herbicides and it may range from several days (e.g. quizalofop-P, mesotrione, MCPA) to as long as several months (e.g. trifluralin, ethofumesate, pendimethalin) (Sekutowski, 2011). It is only a rough indication of the potential persistence of herbicide active substances in soil. Herbicide degradation and translocation under field conditions may occur faster or much slower, since it is result of interactions between chemical properties of the active ingredient itself and moisture content, temperature, absorbing capacity of soil, pH and soil microorganisms. Thus the risk of persistence and translocation of herbicide active ingredients in soil may not be considered only on the basis of one of the parameters e.g. DT_{50}, K_{oc}, R_f, as under field conditions the interactions of all these factors affect

the rate of chemical and biological processes, which in turn determine the behavior of active ingredients of herbicides in the soil environment.

One of the most important herbicide classes are sulfonylureas. These herbicides are extremely active in quite low application rates (less than 100 g of active ingredient per hectare). However, even at low rates, these herbicides can persist in the soil throughout more than one growing season (Moyer, 1995; cit. Bedmar et al., 2006). Sulfonylurea herbicides show a wide range of persistence in both laboratory and field conditions, depending upon soil pH, temperature, and soil moisture. Several authors reported that persistence of sulfonylurea herbicides increased with increasing rate of application, increasing soil pH and decreasing organic matter content (Smith and Hsiao, 1985; Goetz et al., 1989; Castro et al., 2002).

Some sulfonylurea herbicides exhibit longer residual soil activity and may injure following crops. Phytotoxicity to sensitive crops may occur at very low residue concentrations in soil and phytotoxic effects to susceptible plants can be seen for a period several times longer than the DT_{50}. Concentrations at tenths of a nM can cause inhibition of the target enzyme ALS in susceptible crops (Hock et al., 1995). Particularly sensitive are crops from cruciferous family (yellow mustard, oil seed rape), but also other crops such as sugar beet and sunflower are very susceptible (Soukup et al., 2002).

Nicosulfuron is one of the most applicable sulfonylurea herbicides for control of annual and perennial weeds in maize, one of the most frequent pre-crops of sunflower, soybean and sugar beet. Weak volatility and longer persistence of this herbicide have effect to possibility of increased contamination risk to crops in rotation and water systems (Grahovac et al., 2013), more than one season after treatment.

The research in this paper was carried out with the aim of checking presence of nicosulfuron residues in soil.

MATERIAL AND METHODS

Chemicals and solutions

Certificated analytical standard of nicosulfuron (99.1%) was obtained from Dr Ehrenstorfer (Augsburg, Germany). Acetonitrile and methanol, HPLC grade solvents, and H_3PO_4 were purchased from J.T. Baker, Germany. Ultra pure water for HPLC analysis (TKA, Germany) was used. The sulfonylurea herbicide standard stock solution was prepared in acetonitrile at a concentration level of 100 μg/ml, while suitable concentrations of working standards were prepared from the stock solutions by dilution with acetonitrile, achieving concentrations in a range from 2.5 to 20 μg/ml. For matrix-matched calibration, standards were prepared in the same concentrations, by adding standard stock solutions in blank matrix extracts.

Extraction and determination

The extraction and determination procedures had been optimized in our previous study (Lazić and Šunjka, 2014). Nicosulfuron was extracted from homogenized soil samples (10g) with 10 ml of phosphate buffer pH 7.4/methanol (80/20, v/v) solution. The mixture was shaken for 1 min using Vortex and liquid and solid phases were separated by centrifugation at 3000 rpm for 5 min. The extraction process with 10 ml of mixture solution was repeated. Clean-up procedure was done using C18 column. The extract of soil was passed through the column. Nicosulfuron residues were eluted from the column by using a 5 ml of acetonitrile. The eluant was collected in a 10 ml kivet and then concentrated to dryness in nitrogen stream. The residue was dissolved in 1 ml of acetonitrile, ultrasonically homogenized and filtered through a 0.45 μm nylon membrane filter prior to the analysis.

The HPLC-DAD system used consisted of Agilent 1100 Series LC system, equipped with a reversed phase Zorbax Eclipse C18 analytical column of 50 mm × 4.6 mm and particle size 1.8 μm. The external standard method was used for the quantification of nicosulfuron residues.

Sampling

Soil sampling was carried out nine months after nicosulfuron application. Commercial formulation of nicosulfuron was used at the recommended dose, according to the manufacturer's instructions. Samples were collected from surface (0–30 cm) and sub-surface (30–60 cm) soil horizons. In order to ensure representative sample, from a plot on a cultivated field, 5 sub-samples were taken diagonally. The samples were mixed and the average sample of 500 g was formed. In this manner prepared samples were placed into plastic bags, marked and identified, and then transferred to the laboratory. In laboratory prepared average samples that 100 g were air-dried, milled, sieved and analyzed.

RESULTS AND DISCUSSION

Monitoring the trace levels of nicosulfuron herbicide in soil is a challenging task and demands highly efficient, selective and sensitive analytical technique. For selection

of a detection technique the most important criteria in the evaluation is the concentration, at which a given analyte may be found in the tested sample. This problem appears when the herbicide is used once or several times during vegetation in small doses of <50 g/ha (Sadowski et al., 2002, cit. Sekutowski, 2011), as it is the case with products based on nicosulfuron. The method applied in this study is based on reversed-phase liquid chromatography with diode array detection. Method validation ensures analysis credibility. In this study, the parameters accuracy, precision, linearity, limits of detection (LOD) and quantification (LOQ) were tested. The analytical parameters for method determination of nicosulfuron in soil are presented in Table 1 (Lazić and Šunjka, 2014).

The accuracy of the method was evaluated by recovery tests, using the spiked blank soil samples, whereas linearity was determined at five concentrations ranging from 2.5 μg/ml to 20 μg/ml and expressed by correlation coefficient.

The limit of detection (LOD) was determined as the lowest concentration giving a response of 3 times the baseline noise defined from the analysis of control (untreated) sample. The limit of quantification (LOQ) was determined as the lowest concentration of a given herbicide with a response of 10 times the baseline noise. In accordance with SANCO/825/00 rev. 8.1 (SANCO, 2010), the limit of quantification for the determination of pesticides residues in soil should be 0.05 mg/kg.

Table 1. Analytical parameters for HPLC/DAD determination of nicosulfuron in soil

Parameter	Correlation coefficient[a]	LOD mg/kg	LOQ mg/kg	Mean recovery %	Precision RSD%
Nicosulfuron	0.999	0.01	0.05	89.10	<1.06%

[a]Y = ax + b

Presence of matrix effect (SS/E, signal suppression/enhancement) represents influence of soil matrix to nicosulfuron signal. Influence of matrix was determined for whole linear measurement range, from 2.5 to 20.0 μg/ml. Matrix effect was evaluated comparing slope ratio of MMC (matrix matched calibration) and SSC (solvent standard calibration) calibration curves. Regression equation obtained from MMC and SSC were y=25.43x+19.52 and y=26.25x+17.57, respectively. Achieved matrix effect was 96.87%.

Check of the extraction yield was preceded by check of nicosulfuron presence in soil samples that were used as untreated control. The recoveries were calculated by solvent calibration and matrix-matched calibration curves of nicosulfuron. The average value of nicosulfuron extract value from the soil achieved by this method was 89.10% and 86.73%, based on solvent and on matrix-matched calibration curves, respectively, with RSDs less than 2.5%. Obtained values completely fulfil the criteria SANCO/825/00 rev. 8.1 (SANCO, 2010) for pesticide residues in soil (70-120%).

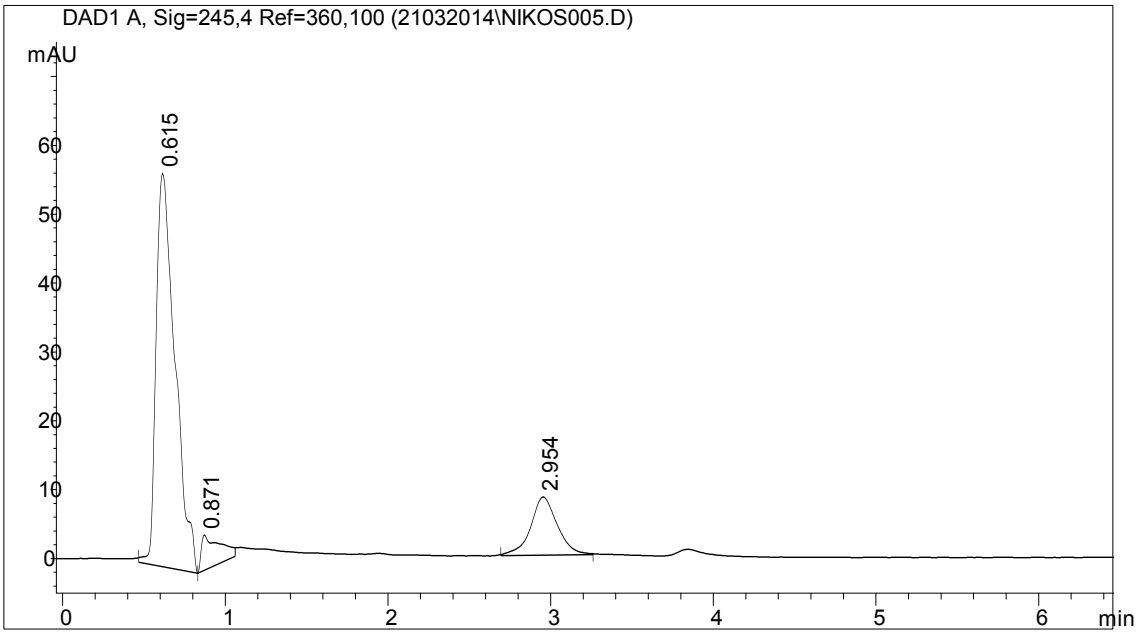

Figure 1. Chromatogram of nicosulfuron in soil sample

The validated method was applied for the analysis of real soil samples. Soil samples were collected from two soil layers, surface (0-30 cm) and sub-surface (30-60 cm) and prepared according to previously described procedure. MMC was used in order to avoid matrix-effect. Average values of nicosulfuron residues in soil samples from surface and sub-surface horizon were 0.05 ± 0.003 mg/kg and 0.05 ± 0.002 mg/kg. According to the Sekutowski (2011), determined content of nicosulforon residues could be harmful for sensitive following crops. Furthermore, the same amount of nicosulfuron found in the both investigated soil layers could be the consequence of agricultural practices applied during autumn.

CONCLUSION

Analytical method described in this study was successfully applied for the determination of nicosulfuron residues in soil samples from surface and subsurface layers. Average values of nicosulfuron residues in soil samples from both horizons were 0.05 mg/kg. The same amount of nicosulfuron found in the both investigated soil layers could be the consequence of cultural practices applied during autumn. Determined content of nicosulfuron residues could be harmful for sensitive following crops. The obtained results indicate that nicosulfuron residues in soil should be monitored to avoid toxicity occurrence in the succeeding crops in crop rotation, especially in years with low amount of precipitation and lower average temperatures.

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PEPPER (*Capsicum annuum*) RESPONSE TO SIMULATED SOIL RESIDUES OF IMAZAMOX

Jelena Gajić Umiljendić*, Ljiljana Radivojević, Ljiljana Šantrić, Marija Sarić-Krsmanović, Tijana Đorđević and Rada Đurović-Pejčev

Institute of pesticides and environmental protection, Banatska 31b, Belgrade, Serbia
**e-mail: pecikos@gmail.com*

ABSTRACT

A bioassay test was conducted to evaluate the sensitivity of pepper to simulated imazamox residues in loam soil with three different levels of moisture (20, 50 and 70% field water capacity – FWC). The soil concentrations of imazamox were established at: 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg a.i./kg soil. The parameters measured 21 days after treatment were shoots and roots fresh weight and root length as well as the content of water soluble proteins. Imazamox caused a growth delay and lower protein content at all levels of soil moisture, and the degree of change depended on the application rate. In plants grown in soil with 20% FWC, root fresh weight showed the highest inhibition, root length showed less sensitivity and there was no statistically significant reduction in shoot fresh weight. In soil containing 50% FWC, only the two highest concentrations caused a significant reduction in shoot fresh weight, while root lenght and root fresh weight were inhibited by ≥50 and ≥100 µg a.i./kg soil, respectively. Root fresh weight was the most sensitive parameter in the soil of 70% FWC, while shoot fresh weight was the least sensitive parameter. Soluble protein contents were lower in all trial variants, but the changes did not depend on herbicide concentration.

Key words: imazamox, pepper, bioassay, loam soil

INTRODUCTION

Imazamox is a selective imidazolinone herbicide applied post-emergence. It inhibits the activity of acetolactate synthase (ALS), the first common enzyme in biosynthesis of the amino acids valine, leucine and isoleucine (Stidham, 1991). Blockage of the synthesis of these three amino acids stops protein synthesis in susceptible plants and reduces translocation of photosynthetic products to the meristem. Disrupted transport of photosynthetic products has a major impact on root growth, which is fully dependent on the energy drawn from the seedling, so that inhibition of root growth is a much more sensitive indicator of the harmful activity of imidazolinones than suppression of seedling growth (Shaner, 1991).

Imidazolinone herbicides act as weak acids, and the presence of both acidic and alkaline functional groups in the molecules of these compounds result in soil pH having a great impact on the availability and transportability of imazamox, so that adsorption in soil increases with pH decrease (Renner et al., 1988; Che et al., 1992; Johnson et al., 2000). Also, there is a high positive correlation between the adsorptiveness of imidazolinones and content of organic matter and clay, and a negative correlation between herbicide transportability and clay content (Stougaard et al., 1990; Mangels, 1991; Undabeytia et al., 2004; Kah and Brown, 2006; Kah et al., 2007). Soil temperature and moisture greatly affect imazamox persistence, and intensive dissipation has been observed under growing temperature accompanied by increasing soil moisture (Vischetti, 1995; Vischetti et al., 2002).

As imidazolinone herbicides are characterized by extended persistance in various media, there are numerous evidences of their effects on the next susceptible crops in crop rotation schemes. Significant damage and yield reduction of sugar beet and rapeseed caused by imazamox residues have been reported by Bresnahan et al. (2002), Pannacci et al., (2006), and Süzer and Büyük (2010). Susceptibility has also been observed in wheat (Deeds et al., 2006), spinach, fennel, green salad and cauliflower (Pannacci et al., 2006), as well as cabbage, tomato and potato (O´Sullivan et al., 1998).

The present study employed an bioassay technique to examine the susceptibility of pepper plants to different concentrations of imazamox in loam soil in which moisture was maintained at different levels.

MATERIAL AND METHODS

Imazamox, technical substance of 95% purity, was obtained from BASF Serbia. Pepper seeds (Editta F1, Enza Zaden) were used in the assay. Loam soil (pH 7.17, humus 3.96%, sand 49.80%, silt 33.40% and clay16.80%) was collected from an area previously never treated with herbicides at the location Zemun Polje. Soil was dug out from 10 cm depth and cleaned from above- and underground plant remains and sifted through 3 mm sieve. The soil was medium calcareous, weakly alkaline and highly humic and with a good supply of total nitrogen and good supply of available phorphorus and potassium. Field water capacity (FWC) was determined by Richard's (1965) method using a pressure plate extractor.

Bioassay experiments were performed by using 250 g of air-dried soil treated with different imazamox concentrations (6.25, 12.5, 25, 50, 100, 200, 400 and 800 μg a.i./kg soil). The soil was uniformly surface-treated and hand-stirred immediately after application, then transferred to pots which were then planted with pepper seeds and watered up to 20, 50 or 70% FWC. Plants grew for 21 days in a controlled-environment chamber under 14 h daylight/10 h darkness and 26°C/day and 21°C/night temperature. Throughout the experiment, soil moisture was constantly maintained at the defined FWC levels. Vegetative parameters – shoot and root fresh weight and root length – were measured as indicators of phytotoxicity, as well as the content of water soluble proteins.

The content of soluble proteins was determined by Bradford's (1976) method. Absorbance of the reaction mix was measured by spectrophotometry at 595 nm wavelength, and afterward, conversion of protein contents (mg/g fresh leaf weight) was conducted.

The effect of imazamox concentrations on these parameters was evaluated using the F-test at 5% significance level. Statistical analysis was performed using StatSoft 8.0. The data were used for a regression analysis to estimate the EC_{50} (effective concentration of imazamox that reduced root fresh weight and root length by 50%) using the software package BIOASSAY97 (Onofri, 1995).

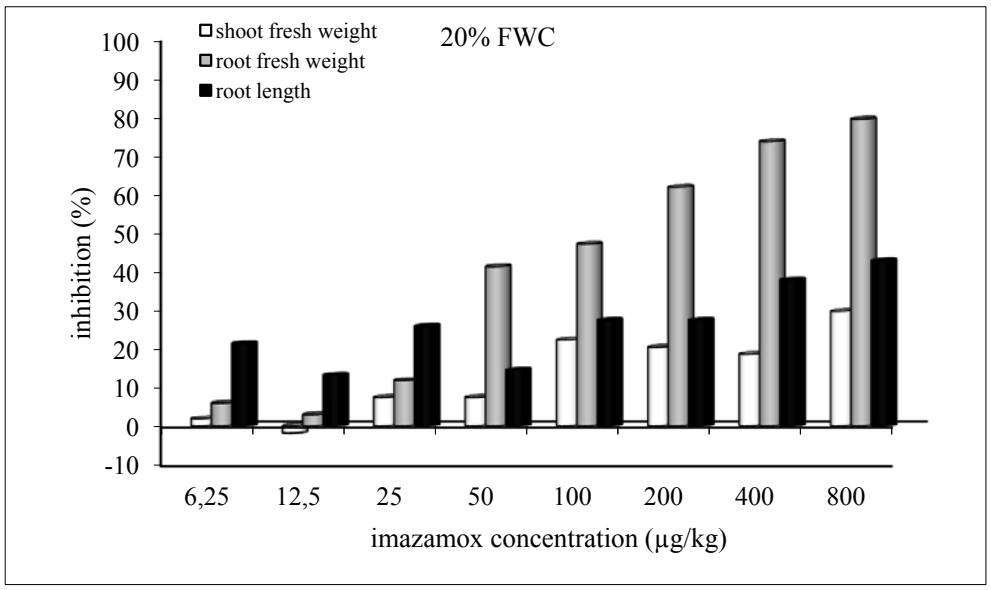

Figure 1. Changes in vegetative parameters of pepper influenced by residual activity of imazamox in loam soil with 20% FWC

RESULTS

Imazamox caused delayed growth and reduced protein contents at all levels of soil moisture, and the degree of changes depended on herbicide application rate. The highest inhibition of root fresh weight was in plants grown in soil with 20% FWC, (Figure 1). A significant reduction was found for concentration of 50 μg a.i./kg 41.18%, while the highest concentration caused 79.41% inhibition. Root length was found to be a less sensitive parameter (13.12-42.76%), while change in fresh weight of seedlings was not statistically significant.

A significant reduction (30-44.29%) in shoot fresh weight in soil with 50% FWC was found only for the two highest concentrations (Figure 2.). Root length was a slightly more sensitive parameter, with inhibition range of 47.57-57.08% for the same treatments. For concentrations ≤50 μg a.i./kg the inhibition observed for root fresh weight was below 15%, while this parameter showed a greater reduction (32.35-64.71%) regarding higher concentrations.

Root fresh weight was the best indicator of sensitivity to imazamox in soil containing 70% FWC, as concentrations from 200 to 800 μg a.i./kg caused 78.72-80.85% inhibition, while root length inhibition for same treatments was significantly lower (41.20-46.99%). Shoot fresh weight was the least sensitive parameter and the highest concentration caused inhibition of only 28.33% (Figure 3.).

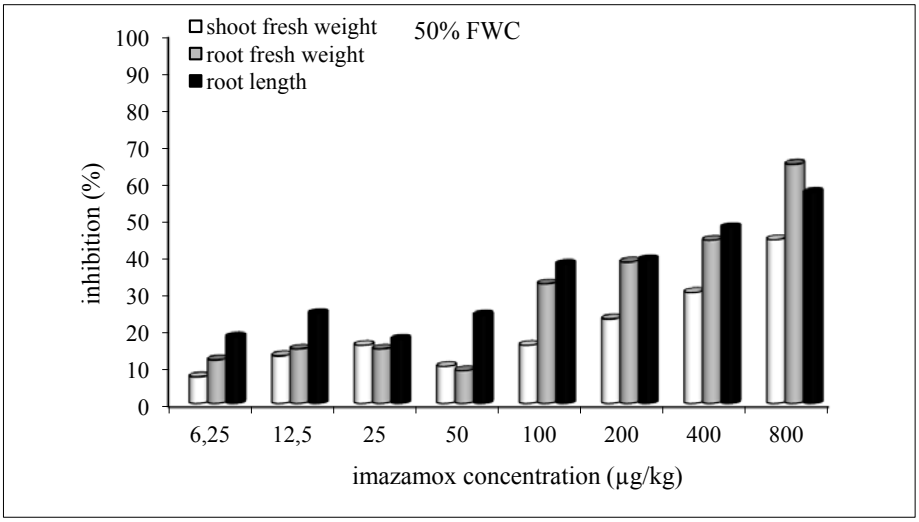

Figure 2. Changes in vegetative parameters of pepper influenced by residual activity of imazamox in loam soil with 50% FWC

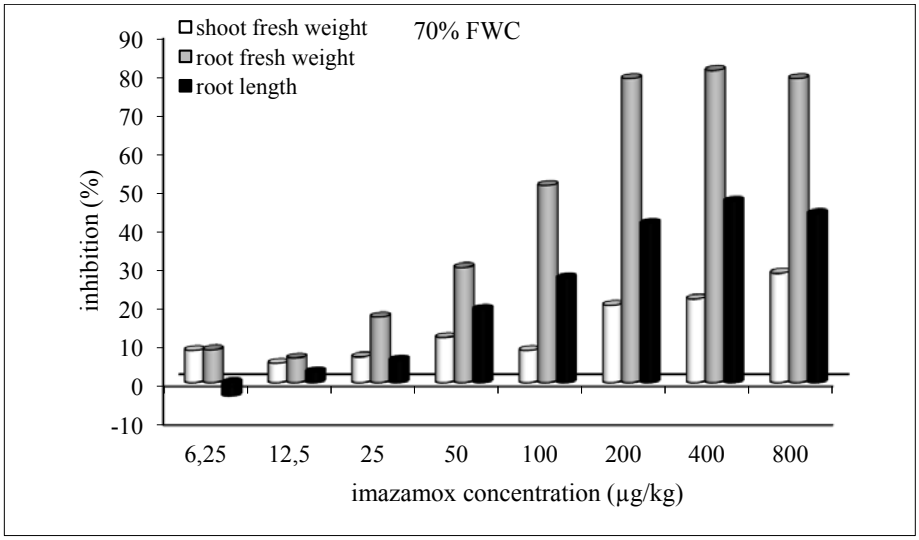

Figure 3. Changes in vegetative parameters of pepper influenced by residual activity of imazamox in loam soil with 70% FWC

Shoot fresh weight was found to be signficantly ($p<0.05$) affected by imazamox and soil moisture, while the interaction of these two factors had no significant effect (Table 1Root fresh weight was significantly affected by both factors and their interaction. Different levels of soil moisture had no effect on root length, while imazamox concentrations and interaction between the herbicide and soil moisture had statistically significant effects.

Regression analysis was used to determine the dependence of pepper roots fresh weight and length on different imazamox concentrations and levels of soil moisture, and EC_{50} values were calculated from that analysis as indicators of plant sensitivity. Considering root fresh weight, the least sensitive were plants grown in soil with 50% FWC (EC_{50} = 170.44 μg a.i./kg), while the EC_{50} values were significantly lower in soil with 20 and 70% FWC, i.e. 66.63 μg a.i./kg and 60.80 μg a.i./kg, respectively. For the root length , plants grown in soil with 50% FWC (EC_{50} = 187.11 μg a.i./kg) were slightly more sensitive, while the corresponding EC_{50} values under 20 and 70% FWC were 194.38 and 221.23 μg a.i./kg.

The effects of different imazamox concentrations on contents of soluble proteins were also examined (Figure 4). That parameter was not found to depend on herbicide concentrations. Under low soil moisture (20% FWC), no inhibition at all or weak inhibition were detected when the higher imazamox concentrations were applied, while the content of soluble protiens in plants exposed to the lowest herbicide concentration was 29.68% lower than in control plants. When soil moisture was set to 70% FWC, the herbicide concentration of 800 μg a.i./kg caused a reduction in proteins of 3.31%, while 6.25 and 12.5 μg a.i./kg concentrations led to 44.96-45.90% reduction. Reduction in protein contents was more evident under 50% FWC, being 35.01-47.19% for the concentrations 12.5-400 μg a.i./kg but the highest concentration again caused a reduction of 10.15%.

Table 1. Two way ANOVA for determining the effects of imazamox and soil moisture on shoot and root fresh weight and root length of pepper

Factor	shoot fresh weight		root fresh weight		root lenght	
	F	p	F	p	F	p
imazamox (concentration)	10.987	0.000000	92.068	0.000000	41.191	0.000000
soil moisture (%FWC)	18.658	0.000000	27.105	0.000000	0.617	0.540891
imazamox *x* soil moisture	0.840	0.638991	6.931	0.000000	2.678	0.001061

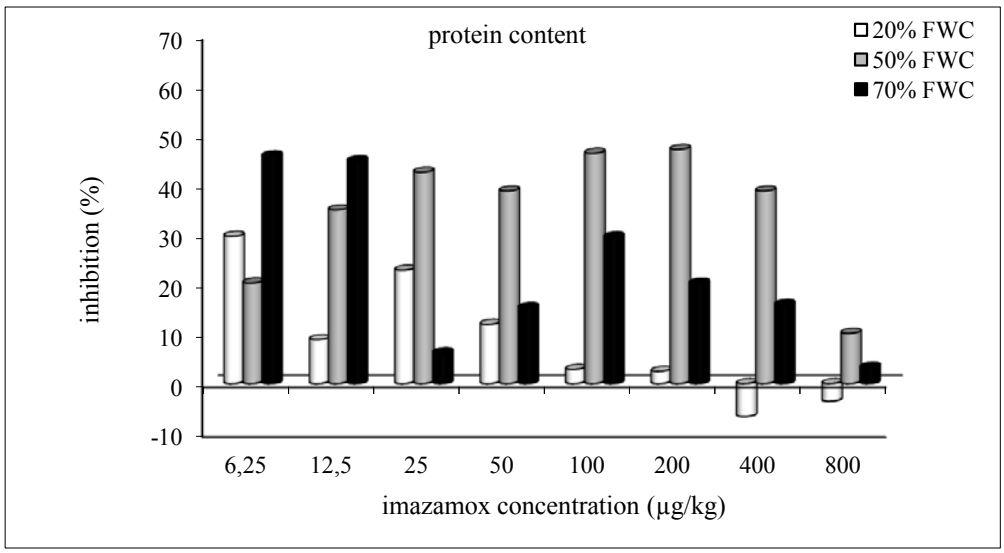

Figure 4. Changes in soluble protein contents in pepper influenced by residual activity of imazamox in loam soil

DISCUSSION

The bioassay in which different imazamox concentrations were applied to loamy soil with different levels of moisture showed a significant susceptibility of pepper plants to herbicide. The observed inhibition showed that root fresh weight was the most sensitive parameter at all levels of soil moisture, while they were slightly lower for root length. Greater sensitivity of the root than shoot of plant species susceptible to imadazolinones has also been reported by Gaston et al. (2002). They found that elongation and growth of pea roots stopped as early as one day after imazethapyr treatment, while shoot growth stopped after three days. Jovanović-Radovanov (2011) also showed in trials of wheat, maize, sunflower, rapeseed, black mustard and sugar beet susceptibility to imazethapyr that the roots of all species involved were more sensitive than their shoots, and also observed a greater sensitivity of fresh weight than length of roots. On the other hand, plant susceptibility to imadazolinones has been assessed in many studies using shoot reduction as a parameter, which is understandable considering that shoots, being the aboveground plant parts, are readily accessable and easy for visual estimation of potential phytotoxicity (Vencill et al., 1990; Alister and Kogan, 2005; Pannacci et al., 2006). The delayed growth that was measured in our study as an inhibition of shoot fresh weight supports those findings.

Decreasing contents of water-soluble proteins were detected in all pots in the present study but that parameter was not found to depend on herbicide concentrations. As the differences were inconsistent with inhibited plant growth, this biochemical parameter was not found to be a reliable parameter for monitoring pepper susceptibility to the residues of imadazolinone herbicides using bioassay techniques.

In studies that examined the effects of residues of imadizolinone herbicides on growth and development of vegetable species, variable data may be found about the sensitivity of the same plant species. O'Sullivan et al. (1998), as well as Greenland (2003), reported that after imazamox treatment of a soybean crop a year earlier, leaf chlorosis and inhibited growth were found in tomato plants but the visual symptoms did not exceed 10% and had no effect on the yield. However, Alister and Kogan (2005) found a significant reduction in the growth and yield of pepper and tomato plants when the two crops were sown in soil in which the combinations imazapyr+imazapic and imazapyr+imazethapyr had been used for treatment a year before. Colquhoun et al. (2003) recommended that an 18-month pause should follow imazamox treatment before pepper, tomato or cucumber could be sown in the same soil. The high degree of inhibition of fresh weight and length of pepper roots found in that study after the activity of the highest (800 μg a.i./kg), as well as several-fold lower concentrations of imazamox, showed that pepper belongs to a group of plants susceptible to that herbicide in loamy soil.

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Plant Protection Society of Serbia, IOBC-EPRS, IOBC-WPRS, Belgrade, 2015, pp. 339 - 342

TESTING OF MICROBIAL ISOLATE SENSITIVITY IN STERILE SOIL AFTER HERBICIDE TREATMENT

Ljiljana Šantrić, Ljiljana Radivojević, Jelena Gajić Umiljendić, Marija Sarić-Krsmanović and Rada Đurović-Pejčev

Institute of Pesticides and Environmental Protection, Banatska 31b, Zemun
ljiljana.santric@pesting.org.rs

ABSTRACT

Herbicides are commonly used in integrated weed management programs. Their application not only controls weed populations, it also affects microbial populations in soil, such as actinomycetes and azotobacters. This study examined the effects of three herbicides (nicosulfuron, metribuzin and glyphosate) on a tolerant actinomycete isolate (14/3) and a tolerant azotobacter isolate (1/13) in sterile soil. The incorporated herbicide concentrations included the recommended field rates, and 10- and 100-fold higher concentrations. Only 15 ml of sterile distilled water was applied in the control. After incubation for 3, 7, 14 and 30 days in treated soil, the growth and abundance of test isolates were checked. The results show that the tested isolates were highy sensitive to glyphosate. On the other hand, stimulating effects of nicosulfuron and metribuzine on the test isolates indicate their ability to break down the molecules of those two active ingredients and use them as a source of biogenic elements and energy for their various processes.

Keywords: herbicides, isolate, actinomycetes, azotobacters, soil

INTRODUCTION

Soil microorganisms participate in processes that are crucial for long-term sustainability of agricultural systems. They occur in soil, in large numbers, as long, as there, is an energy, providing carbon source in it, and they are vital for soil fertility, nutrient cycling, litter decomposition and plant growth. Microorganisms also have an important role in degradation of pollutants in soil, and microbial activity is therefore a significant indicator of soil health (Nannipieri et al., 2003; Pandey et al., 2007). A large number of bacteria occur in soil but their small size is responsible for small biomass. Actinomycetes are a large group of bacteria that grow as hyphae, like fungi, and decompose a variety of substrates, but they are especially important for degradation of complex compounds, such as chitin and cellulose, and are active at high pH levels. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds (Pandey, et al., 2011; Gurung, et al., 2009). Azotobacter is a genus of free-living bacteria that converts atmospheric nitrogen into ammonium, making it available for plant use, and it is active at neutral pH levels. By virtue of these attributes, azotobacter can play nutritional and stimulatory roles and can benefit plants with their manifold actions (Martin et al., 2011; Maurya et al., 2012).

Natural and anthropogenic factors may affect soil microbial activities directly or indirectly. Among the anthropogenic factors, pesticides are of primary importance due to their frequent entry into the soil environment. Long-term and excessive use of pesticides has severe impact on soil ecology that may lead to changes or erosion of the beneficial or plant probiotic soil microflora (Radivojević, et al., 2007; Lo, 2010; Oleszczuk et al., 2014). Herbicides are a major group of pesticides and their intensive use has become a matter of environmental concern, potentially because of their adverse effect on soil microorganisms (Araújo et al., 2003; Šantrić et al., 2014; El Hussein et al., 2012).

The objective of this study was to examine the survival and changing abundance of the selected isolates of actinomycetes and nitrogen fixing bacteria in sterile soil after treatment with nicosulfuron, metribuzin and glyphosate herbicides.

MATERIAL AND METHODS

An experiment was set in the laboratory using soil previously sterilized with 25 kGy for 50 h and conserved for 15 h at the Radiation Unit of the Vinča Institute, Belgrade. After determining the sensitivity of actinomycetes and azotobacters to the investigated herbicides, an actinomycete isolate marked 14/3 and azotobacter isolate 1/31 were selected as the most tolerant, and used for an analysis in sterile soil. The selected actinomycete isolate was incubated for five days in a synthetic medium prepared according to Krasilnikov, and a suspension was then made in sterile distilled water to produce a dilution of 10^4 cfu per 1 ml. Three days after incubation in Fyodorov's medium a suspension of nitrogen fixing bacteria was made to a dilution of 10^3 cfu per 1 ml (Jarak and Đurić, 2006). Sterile soil was supplemented with 5 ml of each diluted test isolate. The herbicides used in this study were nicosulfuron (product Motivell, BASF Company, Germany), metribuzin (product Sencor WG-70, Bayer Crop Science) and glyphosate (product Roundup, Monsanto). The incorporated herbicide concentrations included the recommended field rates, and 10- and 100-fold higher concentrations. Nicosulfuron had three application rates of 0.3, 3.0 and 30.0 mg kg^{-1} soil, while the rates of metribuzin were 12.0, 120.0 and 1200.0 mg kg^{-1}soil, and glyphosate 32.6, 326.0 and 3260.0 mg kg^{-1}soil. An amount of 5 ml of each herbicide concentration was incorporated along with the isolates. Only 15 ml of sterile distilled water was applied to control petri dishes. After incubation for 3, 7, 14 and 30 days in treated soil, the growth and abundance of test isolates were checked.

RESULTS

As shown in Fig. 1a, the profusion of control actinomycetes (isolate 14/3) increased over time. Nicosulfuron reduced the isolate's abundance by 24.2% (log 5.27) seven days after application of its recommeded rate, compared to the control. However, the herbicide had stimulating effects on actinomycete growth in all other trial variants. The isolate showed maximum abundance 14 days after application of the 100-fold concentration, 68.31 x 10^4 g^{-1} soil (log 5.83). Nicosulfuron also had a stimulating effect on the azotobacter isolate (1/31) (Fig. 1b).

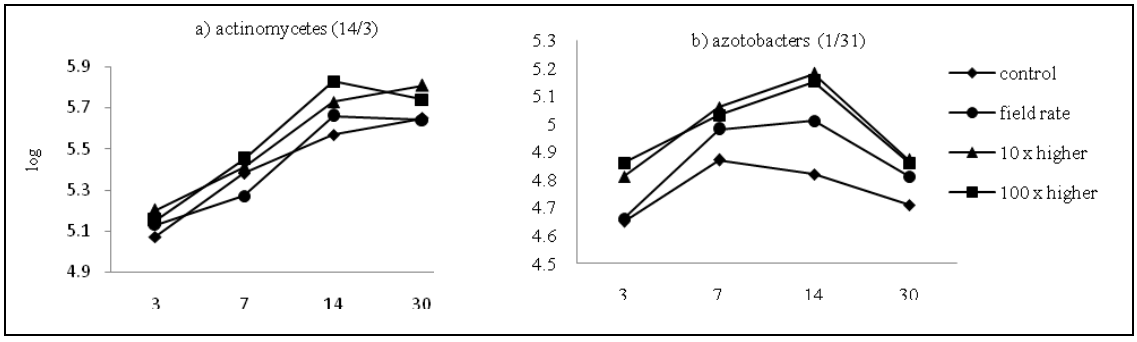

Figure 1. Effects of nicosulfuron on actinomycete and azotobacter isolates

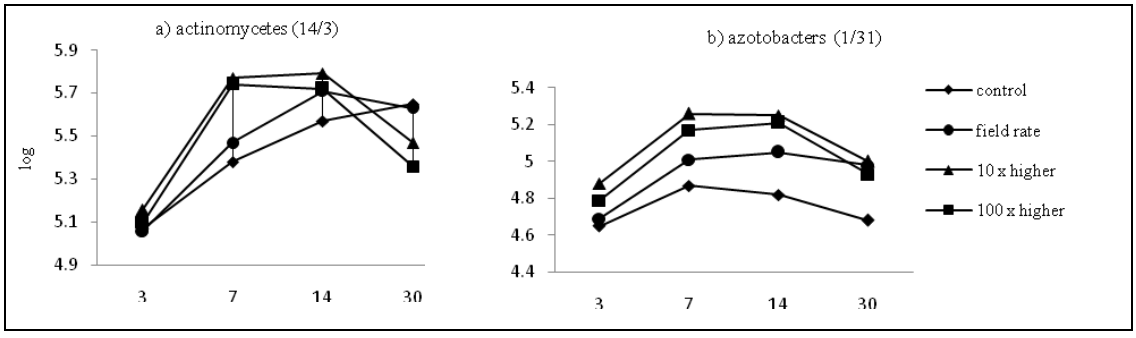

Figure 2. Effects of metribuzin on actinomycete and azotobacter isolates

Its abundance increased, compared to the control, from the seventh day until the end of trial. A maximum was reached on the 14th day and was 154.88 x 10^3 g^{-1} soil (log 5,18). Compared to the control, this value was 125.9% higher.

The actinomycete isolate 14/3 increased its abundance 64.1% under the activity of metribuzin (Fig. 2a) seven and 14 days after application, compared with the control, reaching a maximum of 62.1 x 10^4 g^{-1} soil (log 5.79). Thirty days after herbicide application, the number of actinomycetes decreased 42.4%, compared to the control. Metribuzin also had a positive effect on the azotobacter isolate 1/31 (Fig. 2b). Its abundance increased from the third to the 14th day in all trial petri dishes, reaching a maximum of 183.96 x 10^3 g^{-1} soil (log 5.26), which was 142.3% higher than the control. Minimum value was recorded on the third day and it was 49.71 x 10^3 g^{-1} soil (log 4.69).

The effect of glyphosate on actinomycete numbers is shown in Fig. 3a. The herbicide inhibited the growth of isolate 14/3. Its numbers were reduced in all trial petri dishes throughout the experimental period. The lowest value was recorded 14 days after application and was 15.96 x 10^4 g^{-1} soil (log 5.19). Compared to the control, the number was 57.6% lower. The results (Graf. 3b) show that glyphosate had a positive effect on the azotobacter isolate 1/31 three days after application, when it reached a maximum abundance of 73.35 x 10^3 g^{-1} soil (log 4.86). In all other trial petri dishes, the herbicide had inhibitory effects on the microorganism and reduced its numbers 45.3% against the control 14 days after application, when it was at a minimum of 37.54 x 10^3 g^{-1} soil (log 4.57).

DISCUSSSION

The choice of a laboratory study with sterile soil enabled us to examine more closely the influence of the test herbicides on soil microorganisms, providing us with better understanding of the potential effects of these chemicals on soil microbes. In studying the effects of herbicides on the selected actinomycete isolate (14/3) and azotobacter isolate (1/31) an important aspect was their numbers. It is clear from our data that the herbicides were able to either inhibit or stimulate the two groups of microorganisms, depending on herbicide concentration and period of incubation.

Nicosulfuron and metribuzin showed stimulating effects on the abundance of actinomycetes (isolate 14/3) in all experimental variants from the 7th until the 14th day. From our findings we inferred that the isolate was efficient in utilizing the herbicides and was not sensitive to their toxicity. In a similar study, Latha and Gopal (2010) had observed higher population abundance of actinomycetes after application of field rates of the herbicides pyrazosulfuron ethyl and pretilachlor. They also concluded that the interactions herbicide x concentration and herbicide x days significantly affected actinomycete populations. However, several other studies have reported significant inhibition of a great number of actinomycetes by metribuzin in soil (Zaid et al., 2014; Lone et al., 2014). In contrast to those two herbicides, glyphosate had a negative effect on our isolate throughout the experimental period and decreased its abundance, compared to the control. Conversely, some researchers have reported significant increases in actinomycete populations after the application of glyphosate (Ratcliff et al., 2006; Benslama and Boulahrouf, 2013).

Similar effects were observed on azotobacter (isolate 1/31) after the application of nicosulfuron and metribuzine. The two active ingredients increased the abundance of that isolate, and it reached a maximum 14 days after application. Glyphosate had a positive effect on the isolate in the initial three days, while an inhibitory effect prevailed in all other variants testing the growth and abundance of that group of microorganisms.

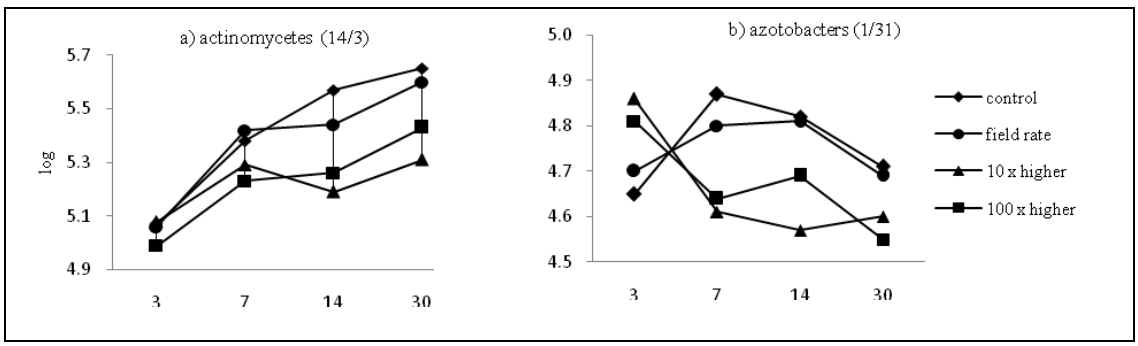

Figure 3. Effects of glyphosate on actinomycete and azotobacter isolates

Khudhur and Askar (2013) had found the herbicide imazethapyr to have no effect on the growth of pure cultures of *Azotobacter chroococcum* and *Azotobacter vinelandii,* while Mrkovački et al. (2002) had found *Azotobacter chroococcum* to be resistant to herbicides and insecticides, while being inhibited by fungicides.

ACKNOWLEDGEMENT

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DETERMINATION OF ACETAMIPRID RESIDUES IN SELECTED VEGETABLE AND FRUIT

Sanja Lazić[1], Dragana Šunjka[1], Pavle Jovanov[2], Nada Grahovac[3], Milica Mojašević[4] and Irena Stojanović[1]

[1]University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, Novi Sad
[2]Institute of food technology, Bulevar Cara Lazara 1, Novi Sad
[3]Institute of field and vegetable crops, Maksima Gorkog 30, Novi Sad
[4]University of Belgrade, Faculty of Agriculture, Nemanjina 6, Zemun
sanjal@polj.uns.ac.rs

ABSTRACT

Increased use of pesticides has resulted in contamination of the environment causing also many associated long-term effects on human health. Therefore, validated analytical methods that produce reliable results for the assessment of pesticide residues in fruits and vegetables are highly needed. The main objective of this study was validation of the method for the analysis of acetamiprid in tomato and determination of its residues after the application at recommended rates under controlled conditions. Obtained results of acetamiprid half-life in tomato are compared with DT_{50} in sweet cherry. For sample pre-treatment QuEChERS procedure was used. Insecticide determination and quantification were performed by HPLC with diode-array detection (Agilent 1100 Series) and Zorbax Eclipse C18 column (50 mm × 4.6 mm internal diameter, 1.8 µm particle size). This method fulfilled validation criteria described in the European Union guidelines (SANCO 12571/2013), by evaluating the accuracy, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ), as well as matrix-effect (ME). The accuracy and precision were satisfactory, showing mean recovery values higher than 80% and precision below 20%, in all cases. The validated method was applied for the analysis of acetamiprid residues in real samples. Half-life of acetamiprid in tomato was 4.33 day and it is quite similar to DT_{50} obtained in the experiment with sweet cherries. On the sixth day after the acetamiprid application residues in tomato were at MRL level, as well as in sweet cherries (according to Serbian MRL, 2010), while the PHI was 14 days.

Key words: acetamiprid, tomato, sweet cherry, residue, DT_{50}

INTRODUCTION

Neonicotinoids are the fastest growing class of insecticides (Muccio *et al*., 2006). They are systemic insecticides that are quite effective in controlling numerous Hemipteran, Thysanopteran, Coleopteran and Lepidopteran species on a wide range of crops, particularly vegetables and fruits (Kim et al., 2003, cit. Park et al., 2010). After the EFSA scientists have identified a number of risks posed to bees by some of neonicotinoid insecticides, the European Commission has excluded from use plant protection products containing clothianidin, thiamethoxam or imidacloprid in crops attractive to pollinators in next two years emphasizing the awareness of potential harmful impact of the neonicotinoids on honeybees

and their products (EU Commission Regulation No. 485/2013 of 24 May 2013; EFSA, 2013). Furthermore, this regulation does not restrict the application of neonicotinoid insecticide acetamiprid {(E)-N1-[(6-chloro-3-pyridyl) methyl]-N2-cyano-N1-methylacetamidine}.

In the Republic of Serbia, acetamiprid is registered for use in tobacco, pepper, cabbage, peach, nectarine and plum for control of aphids, in potato it is used for control of *Leptinotarsa decemlineata*; in tomato for control of *Trialeurodes vaporariorum*; in peas against pea beetle, in onion against leaf miner, in alfalfa against Lucerne leaf-beetle, in apple against leaf aphids, apple moth and apple leaf miner, in pear for control of pear sucker, in cherry and sweet cherries it is used against *Rhagoletis cerasii*, while in vine grape plantations it is used against grape berry moth and grape moth (Sekulić and Jeličić, 2013). Efficiency of products based on acetamiprid led to its intensive use that increased the risk of its residues occurrence in fruits and vegetables. Most of these fruits and vegetables are to a large extent used in fresh conditions without previous thermal treatment. Pre-harvest interval (PHI) for acetamiprid in these cultures is from 14 to 28 days.

Maximum Residue Limits (MRLs) for pesticide residues in fruits and vegetables in our country are stipulated by the Regulations on the maximum allowable quantities of pesticide residues in food and feed for which the maximum allowable quantities of pesticides residues are determined (Official Gazette, RS No. 29/2014). MRL for acetamiprid in tomato established by this Regulation is 0.2 mg/kg and it is entirely in accordance with EU regulations. In relation to sweet cherries, the previous version of the Official Gazette RS from 2010 prescribed the allowable acetamiprid level in sweet cherries of 0.2 mg/kg, while in the new document from 2014, the level is harmonized with EU pesticides database and it is 1.5 mg/kg.

The mainly employed technique for the extraction of pesticides from fruits and vegetables in recent decades is the QuEChERS (Anastassiades et al., 2003). This method involves liquid partitioning with acetonitrile followed by a dispersive SPE clean-up with primary secondary amine and with or without graphitized carbon black (GCB) (Lehotay et al., 2005).

The main objective of this study was validation of the method for the analysis of acetamiprid in tomato and determination of its residues after application at recommended rate under controlled conditions. Obtained results of acetamiprid half-life in tomato will be compared with DT_{50} in sweet cherry, determined in our previous experiment (Lazić et al., 2014).

MATERIAL AND METHODS

Reagents and materials

Acetonitrile HPLC grade and CH_3COOH were obtained from J.T. Baker, Germany. Pesticide standard of acetamiprid was analytical grade (Dr Ehrenstorfer, Augsburg, Germany). Ultra pure water for HPLC analysis (TKA, Germany) was used. Standard was dissolved in acetonitril to make a stock solution of 100 µg/ml. Stock solution was diluted with acetonitrile to make working standard solutions (0.125-1.5 µg/ml) and stored at 4 °C in the dark. For matrix-matched calibration, standards were prepared in the same concentrations, by adding standard stock solutions in blank tomato matrix extracts. Dispersive SP extraction (Cat. No. 5982-5650) and clean-up (Cat. No. 5982-5056) kits for QuEChERS sample preparation were purchased as ready-to-use from Agilent Technologies (USA).

Field trial and sampling

The trial was set up into greenhouse-grown tomatoes at locality Čelarevo. The product based on acetamiprid, MOSPILAN 20 SP with 200 g/kg acetamiprid active substances was used in order to protect tomato from greenhouse white fly (*Trialeurodes vaporariorum*). The solution was prepared at the recommended concentration, according to the manufacturer's instructions (Sekulić and Jeličić, 2013). The samples (around 1.5 kg) were collected before and immediately after application of acetamiprid, and every second day during two weeks (9 samples).

HPLC analysis

HPLC analysis of acetamiprid residues was carried out with an Agilent 1100 Series system equipped with a diode-array detector (Lazić et al., 2014). HPLC determination was conducted using an Agilent Zorbax Eclipse C18 column (50 mm × 4.6 mm internal diameter, 1.8 µm particle size). Mobile phase was acetonitrile and 1.5% CH_3COOH in ultrapure water (30/70) in an isocratic elution at the flow rate of 1.0 ml/min. The column temperature was maintained at 25 °C. Volume of 2.5 µl was injected with auto sampler. Detector wavelength was 254 nm.

Extraction and clean-up procedure

For the extraction of acetamiprid from tomato QuEChERS method (EN 15662 version 2.2, 2008) was used. At 10 g homogenized sample, 10 ml of acetonitrile

was added and vigorously shaken for 1 min. After that, a mix of buffered salts was added and again shaken for 1 min and centrifuged for 5 min at 3000 rpm. Aliquot of 6 ml of the upper acetonitrile layer was transferred to 15 ml centrifuge tube containing the sorbent, mixture of primary-secondary amine (PSA) and magnesium sulphate. The tube was vigorously shaken for 1 min and then centrifuged at 3000 rpm for 5 min. An aliquot of the final upper layer was evaporated to dryness, dissolved in 1 ml of acetonitrile, filtered through a 0.45 μm membrane filter and transferred into an autosampler vial for analyses.

Validation of the analytical method

The method for quantitative analysis of acetamiprid in tomato was validated in terms of accuracy, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ), as well as matrix-effect (ME), in accordance with Document SANCO/12571/2013.

RESULTS AND DISCUSSION

Neonicotinoid's residues in different matrices are usually determined by liquid chromatography LC with diode array detection (Obana et al., 2002; Lazić et al., 2012, 2013), since direct analysis by gas chromatography is unsuitable due to their low volatility and high polarity. Nowadays, they are determined by LC-MS-MS and LC/TOF-MS in vegetables and fruits (Ferre et al., 2005; Jansson et al., 2004; Park et al., 2011; Lazić et al., 2014a).

The validation of the chromatographic method for determination of acetamiprid was carried out using HPLC/DAD under previously described conditions. The linearity of the calibration curve was examined using five calibration solutions prepared in acetonitrile.

The calibration curve was obtained by plotting peak areas in 'y' axis against concentrations of the pesticide in 'x' axis within the investigated range (Figure 1) of concentrations. Each solution was injected in triplicates. The linearity was good with a high correlation coefficient of R^2=0.999.

The obtained LOD and LOQ were 5 μg/kg and 14 μg/kg, respectively. This method provides detection and quantification limits lower than the MRL established by the EU (Europe Commission, 2010) and the Serbian Regulations for acetamiprid in tomato of 0.2 mg/kg. The precision values for the method, expressed as repeatability (relative standard deviation-RSDr) of peak area (n=6), was less than 1.0%.

For determination of acetamiprid recovery, untreated tomato samples (10 g) were spiked with acetamiprid at three concentration level, from LOQ-0.3 mg/kg. Samples were allowed to stand for 30 min, prior to extraction

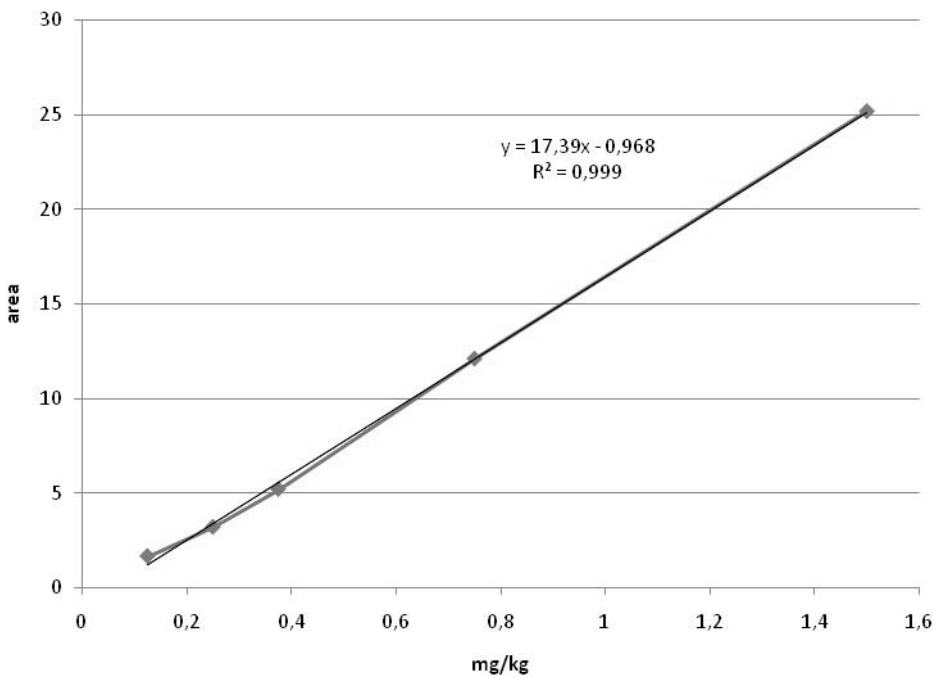

Figure 1. Calibration curve for acetamiprid standards in acetonitrile

by QuEChERS method. Figure 2 presents chromatogram of acetamiprid in tomato matrices.

The mean recovery of acetamiprid in tomato was 96.7±1.1%. According to the EU validation guideline for pesticide residues, mean recovery values should be within the range of 70–120% at each spiking level with acceptable RSDs≤20%. Our previous studies validated the method of the insecticide acetamiprid determination in sweet cherries. The average recovery was 85.4% with RSD=2.5% (Lazić et al., 2014). Matrix effect (suppression or enhancement) was evaluated through the matrix effect percentage (%ME) calculation. This calculation was carried out in accordance with the literature report (Ferrer et al., 2005) as the percentage of the difference between the slopes values of the matrix-match calibration curve and the solvent one. The effect of tomato matrix to acetamiprid signal was 98.04% and there was no observed matrix-effect. In this study, no matrix effect is considered as the values of %ME are into the accepted values (100±20%).

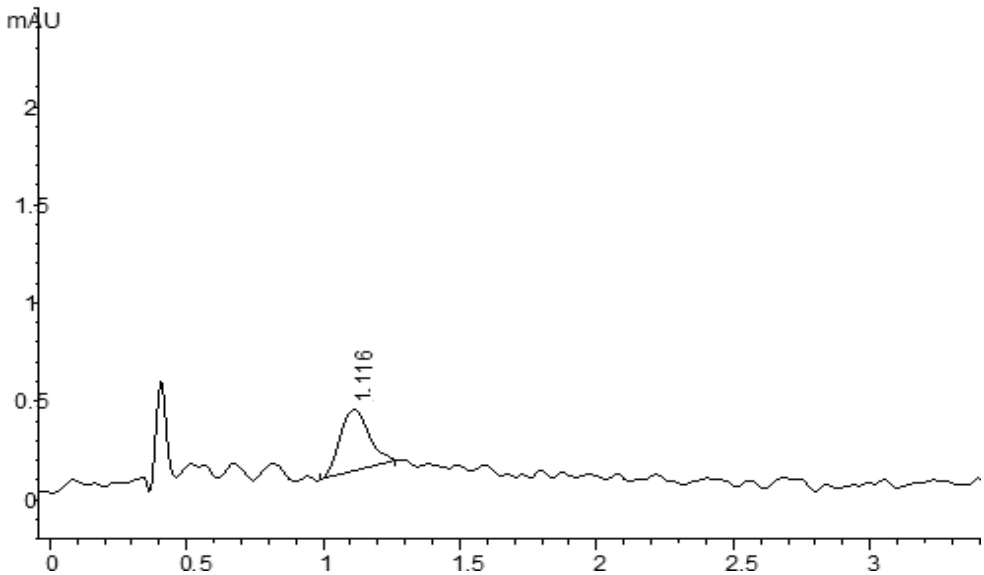

Figure 2. Chromatogram of acetamiprid in tomato sample (0.1 mg/kg)

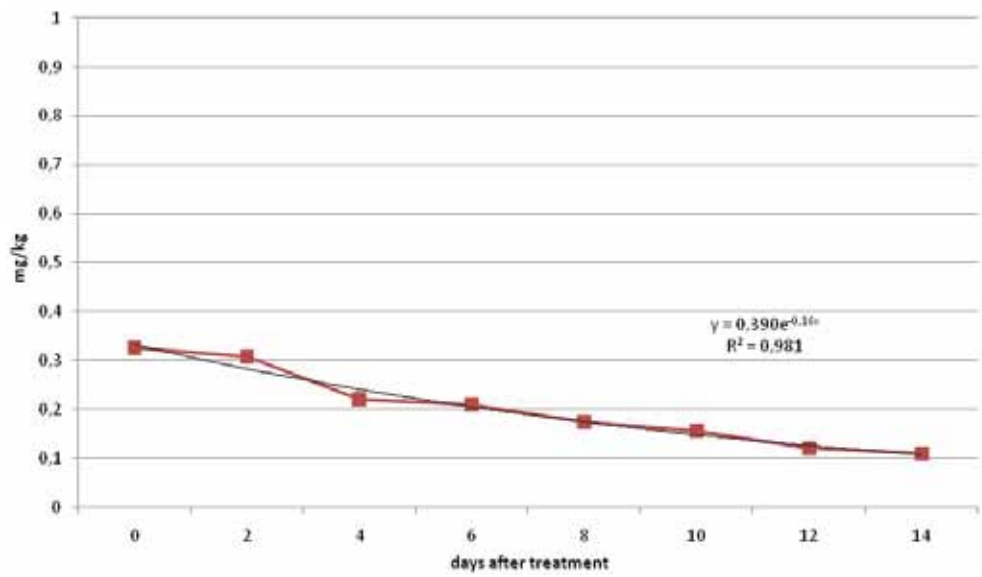

Figure 3. Dissipation of acetamiprid residues in tomato samples during 14 days period

The validated method was applied for the analysis of acetamiprid residues in real tomato samples. Matrix-match calibration was used for the quantification of acetamiprid residues in tomato samples. Pre-harvest interval for acetamiprid in tomato set by Serbian Regulations is 14 days. The maximum residue level of acetamiprid, 0.33 mg/kg, in tomato samples was detected immediately after the application. Two and four days after the application, residues of acetamiprid were 0.31 mg/kg and 0.22 mg/kg, respectively. Six days after the application, acetamiprid content in tomato was at MRL level of 0.2 mg/kg (Figure 3).

In this study half-life (DT_{50}) was calculated from the exponential equation (Figure 3). DT_{50} of acetamiprid in tomato samples obtained in this study was 4.33 days. Half-life for the acetamiprid in medium late variety of sweet cherry, achieved in our previous experiment, was 3.15 days (Lazić et al., 2014).

CONCLUSION

In this study, the method for the determination of acetamiprid residues in tomato using QuEChERS procedure followed by high performance liquid chromatography, was validated. The proposed method proved to be an efficient and sensitive method for the determination of acetamiprid in tomato. Half-life of acetamiprid in tomato was 4.33 day and it is similar to DT_{50} obtained from our experiment in sweet cherries. At 6^{th} day after the acetamiprid application residues were at MRL level. These results are similar to the results from the experiment with sweet cherries. On the sixth day after the acetamiprid application residues in tomato were at MRL level, as well as in sweet cherries (according to MRL from 2010), while the PHI was 14 days.

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DETERMINATION OF METRIBUZIN IN PLANT MATERIAL BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Gorica Vuković[1], Bojana Špirović[2], Vojislava Bursić[3], Jelena Vlajković[1] and Katarina Jovanović-Radovanov[2]

[1]Institute of Public Health of Belgrade, Belgrade, Serbia, [2]Faculty of Agriculture, University of Belgrade, [3]Faculty of Agriculture, University of Novi Sad, Serbia
E-mail: gorica.vukovic@zdravlje.org.rs

ABSTRACT

The goal of our study was the development and validation of the method for the determination of metribuzin residues in alfalfa (*Medicago sativa*). The metribuzin was extracted from plant samples using an extraction procedure based on the QuEChERS methodology modified for pigmented vegetables with the clean-up being modified by graphitized carbon black (GCB) sorbent. LC-MS/MS method with positive electron spray ionization (ESI) was used for the determination of metribuzin residues. The separation of the compounds from the plant extracts was achieved using reverse phase Zorbax C18 column (50mm×4.6mm i.d.) with 1.8 µm particle size. The identification and confirmation of metribuzin were based on the retention time and two typical monitoring transitions (MRM). The optimized analytical conditions were evaluated in terms of recovery, reproducibility, limit of quantification (LOQ) and linearity. The matrix influence on linearity and recovery and its effects on ionization was evaluated. The calibration range was from 0.010 to 0.100 mg/kg. The recovery was investigated at three levels of 0.01, 0.05 and 0.10 mg/kg and ranged from 84.3 to 93.2% (RSDs 4.47-5.37%).

Key words: Metribuzine residues, QuEChERS, Alfalfa, LC-MS/MS

INTRODUCTION

Alfalfa (*Medicago sativa*) is one of the most important forage crops in our country. It has the highest yield potential and one of the highest feeding values of all adapted perennial forage legumes. Due to the rich and variable genetic base it has good adaptability to different environment conditions and wide area of growing. Now it is cultivated in more than 80 countries on every continent of the globe in the area exceeding 35 million ha (Radović et al., 2009). Alfalfa is grown over a wide range of sod and climatic conditions and plays an important role in crop rotation through its positive effects on sod fertility, sod structure and reduced sod erosion.

Modern agriculture depends, to a large extent, on the use of herbicides in order to control the weeds that compete with the crops. Metribuzin is a selective systemic herbicide used for pre- and post-emergence control of many grasses and broad-leaved weeds in soya beans, potatoes, tomatoes, maize, alfalfa and cereals at 0.07-1.05 kg a.i./ha (Huertas-Perez et al., 2006). On our market there is one WP formulation of this herbicide (Mistral, 700 g/kg) registered for the use in alfalfa (Sekulić and Jeličić, 2013). This herbicide is efficient for the different weed species: *Amaranthus retroflexus*, *Capsella bursa-pastoris*, *Centaurea cyanus*, *Chenopodium album*, *Digitaria spp.*, *Poa annua etc.* (Janjić, 2005). Metribuzin belongs to the group of triazinone herbicides with water solubility of 1.05 g/L (20 °C) (MacBean, 2012).

The aim of this study was to develop and validate the QuEChERS single method for the determination of metribuzin in alfalfa using liquid chromatography tandem mass spectrometry – LC-MS/MS.

MATERIAL AND METHODS

Chemicals and apparatus

All solvents used were of chromatography grade and were obtained from Merck (Darmstadt, Germany). The certified pesticide analytical standard of metribuzin (99.5 %) was purchased from Dr. Ehrenstorfer (Augsburg, Germany) and the internal standard (IS) carbofuran-D3 (99.7%) was purchased from Pestanal, Fluka (Germany). An internal standard was used in the concentration of 10 μg/mL in acetonitrile. The stock standard solution was prepared by dissolving the analytical standard of metribuzin in acetonitrile (1mg/mL), while the working standard was obtained by diluting the stock standard with acetonitrile resulting in the final mass concentration of 1 mg/mL. Magnesium sulphate, disodium hydrogencitrate sesquihydrate, trisodium citrate dihydrate, sodium chloride and formic acid (analytical reagent grade) were purchased from Fisher Scientific UK (Loughborough, UK). Bondesil primary secondary amine (PSA, 40 μm) and graphitized carbon black (GCB) sorbent were obtained from Agilent Technologies (Australia Pty Ltd). For LC analysis, an Agilent 1200 (Agilent Technologies, USA) HPLC system with a binary pump was used. This was equipped with a reversed-phase C18 analytical column of 50×4.6 mm and 1.8 mm particle size (Zorbax Eclipse XDB-C18, Agilent). The mobile phase was methanol (solvent A) and Milli-Q water (solvent B), both containing 0.1% formic acid in gradient mode, with the flow rate of 0.6 mL/min. The elution program was started with 50% B. It was linearly decreased to 30% B in 12 min and held constantly for 3 min. The stop time was 15 minutes with the post run of 3 minutes. The injection volume was 5 μL.

For the mass spectrometric analysis, an Agilent 6410 Triple-Quad LC/MS system was applied. Agilent MassHunter B.04.00 software was used for the data acquisition and processing. The analysis was performed in the positive ion modes. The ESI source values were as follows: drying gas (nitrogen) temperature 300 °C, vaporizer 200 °C, drying gas flow rate 5 L/min, nebulizer pressure 50 psi and capillary voltage 2500 V. The detection was performed using the multiple reactions monitoring (MRM).

Validation parameters

Limit of detection (LOD), limit of quantification (LOQ) and linearity

The evaluation of the linearity was done based on the injections of standard solutions prepared in the mobile phase and also in the extract of blank alfalfa samples, at the concentrations of 0.002, 0.010, 0.1, 0.050 and 0.1 μg/mL, with the addition of IS.

The LOD was estimated from the chromatogram of the lowest level of calibration using the Agilent MassHunter software (Agilent Technologies, Data Acquisition for Triple Quad B.03.01) for those concentrations that provide a signal to noise ratio of 3:1.

The LOQ was based on the accuracy and precision data, obtained via the recovery determinations and was defined as the lowest validated spike level which meets the requirements of a recovery within the range of 70 – 120% and a RSD ≤ 20 % (SANCO/12571/2013. The LOQ was determined at 0.01 mg/kg, in consideration of MRL (0.1 mg/kg) (EU Regulation 396/2005).

Recovery

The main goal of the recovery experiments is to determine the method accuracy via the comparison of the real concentration of a pesticide, measured by performing the complete procedure, with the known pesticide concentration initially added to the matrix. The method precision is expressed as the repeatability (RSD, %) of the recovery determinations at three different spiking levels (0.01, 0.05 and 0.10 mg/kg).

Sample preparation

The metribuzin was extracted from alfalfa samples using an extraction procedure based on the QuEChERS methodology (Anastassiades, 2003). For the alfalfa extract, the amount of 2 g of fine homogenised sample was used. The samples were then mixed with 10 mL of water before the extraction. It was followed by adding 100 μL of IS solution and by the extraction with 10 mL of MeCN. After extracting on vortex mixer for 1 min, 6.0 g of magnesium sulfate anhydrous, 1.5 g of sodium chloride, 1.5 g of trisodium citrate dihydrate and 0.75 g of disodium hydrogencitrate sesquihydrate were added and the mixture was shaken vigorously for 1 min and after that centrifuged for 5 min at 3000 rpm. After the centrifugation 1 mL of supernatant was transferred into a clean-up tube containing 900 mg of $MgSO_4$, 150 mg of GCB and 150 mg of PSA. After the centrifugation for 5 min at 4500 rpm, 0.5 mL of supernatant was evaporated to dryness and reconstituted in 0.5 mL of mobile phase.

RESULTS

The summary of MRM transitions and MS operating parameters selected for the analysis of metribuzin and carbofuran-d3 in ESI positive mode are given in Table 1.

Table 1. MRM transitions of metribuzin and carbofuran-D3.

Pesticide	Formula	M (g/mol)	Precursor ion	Product ion	Frag (V)	CE (V)
Metribuzin	$C_8H_{14}N_4OS$	214.28	215	187	100	17
			215	84	100	20
Carbofuran-d3	$C_{12}H_{12}D_3NO_3$	224.27	225.1	165	94	10
			225.1	123	94	22

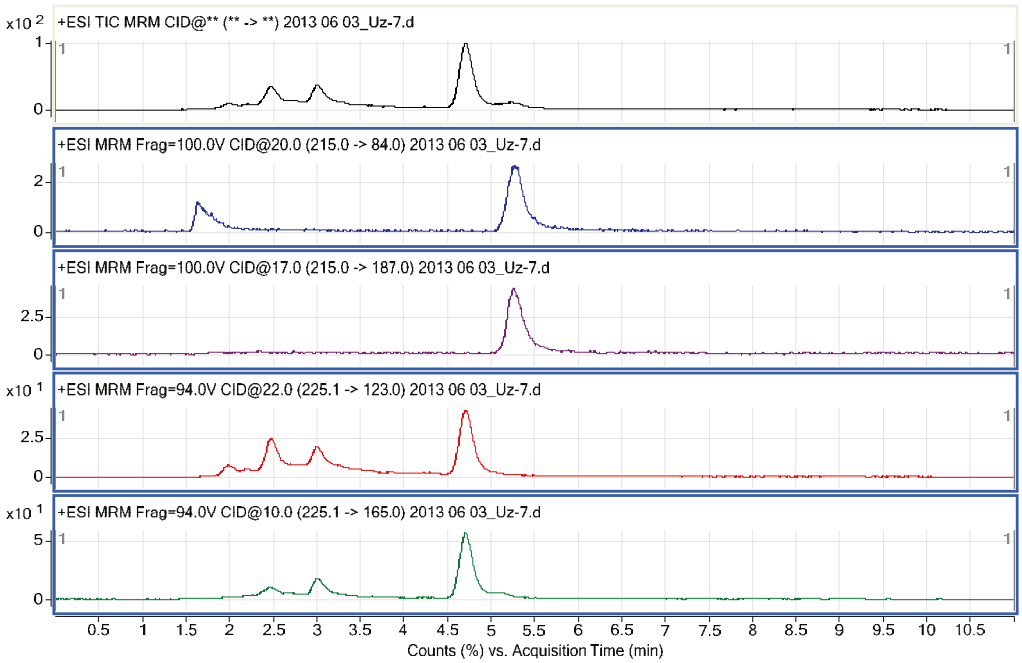

Figure 1. LC-MS/MS chromatogram of alfalfa sample

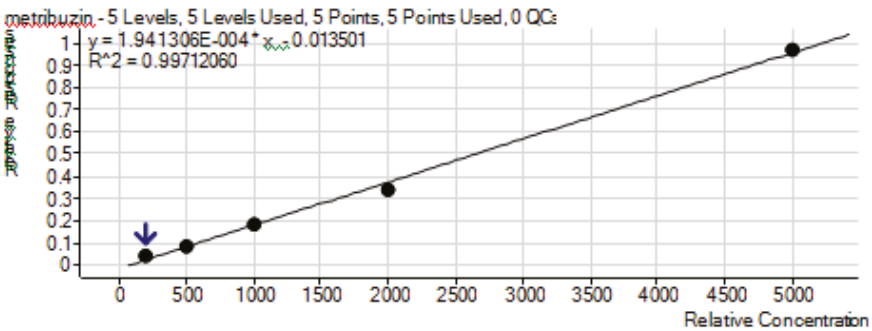

Figure 2. Calibration curve for metribuzin in solution

Chromatogram of MRM identification of metribuzin in real alfalfa sample is given in Figure 1.

Calibration, LOD and LOQ

The alfalfa control based matrix used for the calibration and for the recovery studies was analyzed to verify the absence of metribuzin before performing the analysis. The calibration curves based on matrix-matched standards were obtained at the concentration levels from 0.002-0.10 μg/mL at five levels. The matrix effect was observed comparing the slopes obtained for the calibration curves of matrix-matched standard, with the slope calibration curve in mobile phase. The matrix effect was 1%. Good linearity was achieved, with the coefficient of determination (R^2) better than 0.99 (Figure 2 and 3).

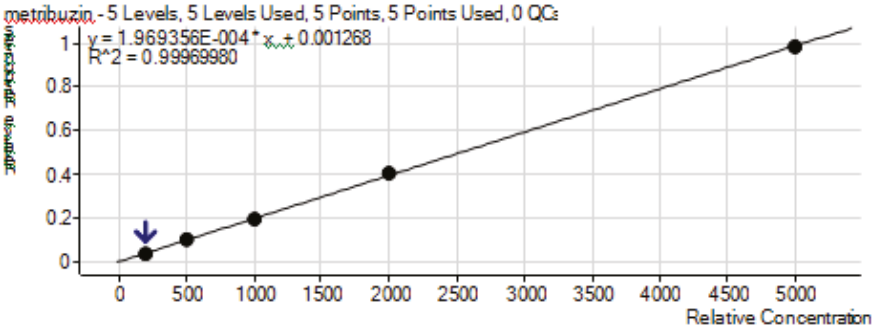

Figure 3. Calibration curve for metribuzin in matrix

Recovery

The recovery studies were performed with the fortification experiments at three levels (0.01, 0.05 and 0.10 mg/kg) in three replicates with the addition of internal standard carbofuran-d3. The pesticide–free samples were spiked before the QuEChERS method and analyzed as previously described. The average recovery was 89.7±4.98% (Table 2). The precision was assessed in terms of repeatability at 10 mg/kg.

A good repeatability (n = 6), with RSDs of 4.98% was obtained and it was calculated through the recovery.

Table 2. Recoveries in alfalfa sample

Concentration (mg/kg)	Replicates			Average recovery (%)	RSD (%)
	1	2	3		
0.01	86.9	79.4	86.7	84.3	4.47
0.05	94.2	91.8	88.6	91.5	5.11
0.10	94.7	90.1	95.1	93.2	5.37

DISCUSSION

The validated method which uses a liquid chromatography tandem mass spectrometry (LC-MS/MS) provides a very high sensitivity, good reproducibility, appropriate linearity and can be applied with a high reliability to the analysis of investigated herbicide residue in real alfalfa samples. The validation parameters with the low LOQs of 0.01 mg/kg confirm that the method is suitable for the determination of pesticide residues in real alfalfa samples according to the regulations of the Serbian and EU MRLs.

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DETERMINATION OF PESTICIDE RESIDUES IN WATERMELONS BY LC-MS/MS

Vojislava Bursić[1], Gorica Vuković[2], Ranko Čabilovski[1], Tijana Zeremski[3], Marko Ilić[1] and Renata Baličević[4]

[1]Faculty of Agriculture, University of Novi Sad, Serbia
[2]Institute of Public Health of Belgrade, Belgrade, Serbia
[3]Institute of Field and Vegetable Crops, Novi Sad, Serbia
[4]Faculty of Agriculture, Josip Juraj Strossmayer University in Osijek, Croatia
E-mail: bursicv@polj.uns.ac.rs

ABSTRACT

The LC–MS/MS was applied for the detection of pesticide residues in watermelons. Since watermelons are predominantly used as fresh food and to a lesser extent in food processing there is a justified concern that, due to treatments, they can contain pesticide residues above the maximum residue levels – MRLs. The pesticide extraction was carried out by QuEChERS method. The samples were tested regarding the content of 55 pesticides with the carbofuran-D3 as internal standard. The linearity was studied in the range of 0.01–0.50 mg/kg and the determination coefficients (R^2) were higher than 0.99 for all the investigated pesticides. The calibration was performed as matrix calibration, by means of spiking the calibration samples before the extraction and preparing them in the same way as the test samples. The recovery data were obtained by spiking blank samples at three concentration levels (0.01, 0.05 and 0.1 mg/kg) yielding recoveries in the range of 61.0–114.2% with the relative standard deviation (RSD) less than 13%. The limits of quantification (LOQs) were established as 0.01 mg/kg. The multiple detections were confirmed in the analysed samples. The most frequently detected pesticides were carbendazim, acetamiprid, tefluthrin and fenpropimorph. In only one sample the concentrations of carbendazim and tefluthrin were above the MRLs.

Key words: Pesticide residues, QuEChERS, watermelons, LC-MS/MS

INTRODUCTION

The watermelon (*Citrulus vulgaris* sin. *Citrulus lanatus Thumb.*) is a sweet, juicy, rich in β-carotene (Đurovka and Ilin, 2002) and very nutritious fruit, that is packed with some of the most important antioxidants in nature. In addition to vitamin C and A, the watermelon harbors lycopene, an efficient oxygen radical scavenger, which can protect against chronic diseases such as cancers, cardiovascular diseases, and osteoarthritis inflammation (Park et al., 2010). Since watermelon is farmed primarily *via* protected and successive cultivation techniques, it is far more susceptible to physiological disorder and damage due to pests and diseases than are plants raised under usual cultivation conditions (Nguyen et al., 2008). A number of pesticides need to be used to control pests and diseases in order to increase watermelon production. On our market there are 7 compounds registered for the use in watermelon protection out of which 1 is an insecticide, 4 are herbicides and 4 fungicides (Sekulić and Jeličić, 2013). Additionally, many chemicals deriving from indirect sources such as soil, contaminated with agro-inputs, drift from adjoining crop fields, *etc. and* may contaminate the edible inner part of the

watermelon, and may affect human health (Park et al., 2010). Since watermelons are predominantly used as fresh food and to a lesser extent in food processing there is a justified concern that, due to treatments, they can contain pesticide residues above the maximum residue levels – MRLs (Bursić et al., 2014).

The health safety of food is of great significance for consumers, food industry and economy (Jevšnik et al., 2008). Thus our county adapted the MRLs values to the current MRLs in the European Union (Off. Gazette RS 29/2014; Regulation EC No 396/2005).

Therefore, to protect human health and control the environmental pollution, sensitive and efficient analytical methods for the determination of pesticide residues at trace levels are desirable (Wang et al., 2011). Nowadays, many analytical methods reported in the literature for the determination of pesticides involve gas chromatography (GC) equipped with most commonly used detectors such as electron capture detectors (ECD), nitrogen phosphorus detectors (NPD) or mass spectrometers (MS) which have been used broadly for many years to monitor volatile and thermally stable pesticides. The liquid chromatography-mass spectrometry (LC-MS) is an ideal technique for the analysis of residues of non-volatile and thermally unstable pesticides. It has been recently reported that LC-MS/MS is capable of analyzing pesticide multiresidues more efficiently than LC-MS (Park et al., 2010). The LC/MS-MS method has high selectivity and sensitivity, simplicity of sample cleanup, and easy and reliable indentification and quantification, even at trace levels of pesticides (Vuković, 2012).

Nevertheless, there are some difficulties for pesticide residues direct determination due to their low concentration in most cases. So, to obtain accurate and sensitive results, the determination of the pesticides is usually accomplished by many preliminary steps like sampling, extraction, and clean-up for interference removal and analyte concentration before chromatographic analysis (Wang et al., 2011). The trends in recent years have been directed towards the decrease in sample amounts for the analysis with the approach which is safe and less harmful to the environment and at the same time implies a quicker, simpler method for sample preparation with simultaneously providing high recovery and good precision (Bursić et al., 2013). Anastasiades et al. (2003) developed a quick, essential, cheap, efficient, robust and safe method (QuEChERS) so as to overcome the limitations of the existing preparation methods.

To evaluate the negative effects of pesticides in watermelons and to ensure the consumers safety, the validated multiresidue LC/MS-MS method was used for the detection of pesticide residues. The pesticide extraction was carried out by the most promising sample preparation techniques QuEChERS (Vuković et al., 2012; Bursić et al., 2014). That is why in this study the purpose was to use QuEChERS method for the extraction and LC-MS/MS for the detection of 55 pesticides in watermelons in the control of human food safety.

MATERIAL AND METHODS

Materials

All solvents used were chromatography grade and were obtained from J.T. Baker (Deventer, Netherlands). Certified pesticide analytical standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), most of them of purity ≥98%. The internal standard carbofuran-D3 (99.7%) was purchased from Pestanal, Fluka (Germany).

QuEChERS Extract Tubes, EN Method, part No: 5982-5650 and Dispersive SPE 15 mL (High pigmented), part No: 5982-5356 were purchased from Agilent Technologies (USA).

Sample preparation

The samples were taken in accordance with the Regulation on methods of food sampling and testing aimd at the determination of plant protection products residues in food (Off. Gazzete RS No 110/2012) which defines the sampling methods and the minimum amount of laboratory samples. Three average samples of watermelon at the stage of technological maturity were taken from the fields. The samples were put into polyethylene bags and immediately transferred to the laboratory. On arrival each sample was homogenyzed and kept in a deep freeze at the temperature of -18 °C till being analyzed.

The extracts were obtained using the acetonitrile-based QuEChERS sample preparation technique (Figure 1.). The basic samples of waterelons, were collected from various field in Vojvodina. The sampling was carried out at the end of July 2013. All the samples were kept in polyethilene black bags in deep-freeze until being analyzed.

Analytical determination

Agilent 1100 Series HPLC system with Zorbax XDB C18 analytical column of 50×4.6mm and 1.8 mm particle size (Agilent Technologies) column was

used. For LC analysis, an Agilent 1200 HPLC system with a binary pump was used. For the mass spectrometric analysis, an Agilent 6410B Triple-Quad LC/MS system was used. Agilent MassHunter Workstation Software version B.04. QQQ Agilent Technologies, 2011 were applied for method development and data acquisition.

Validation: The method was validated according to SANCO/12571/2013. The limit of detection - LOD was determined as the lowest concentration giving a response of three times the average baseline. The ratio signal/noise in the obtained chromatograms for the LOD was calculated MassHunter Qualitative Software. The linearity was checked using matrix matched standards (MMS) at concentrations of 5.0, 10.0, 25.0, 50.0 and 100.0 ng/mL. The recovery was checked by enriching 10 g of a blank sample with the mixture of pesticide standard of 10 mg/ml in the amount of 100 and 50 µL (final mass concentration 0.10 and 0.05 mg/kg) and with the mixture of pesticide standard of 1 mg/mL in the amount of 100 µl (final mass concentration 0.01 mg/kg) with the addition of the internal standard carbofuran-D3.

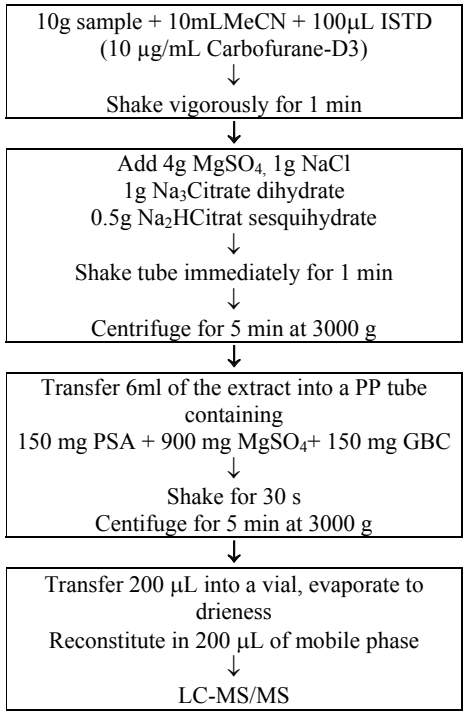

Figure 1. QuEChERS extraction

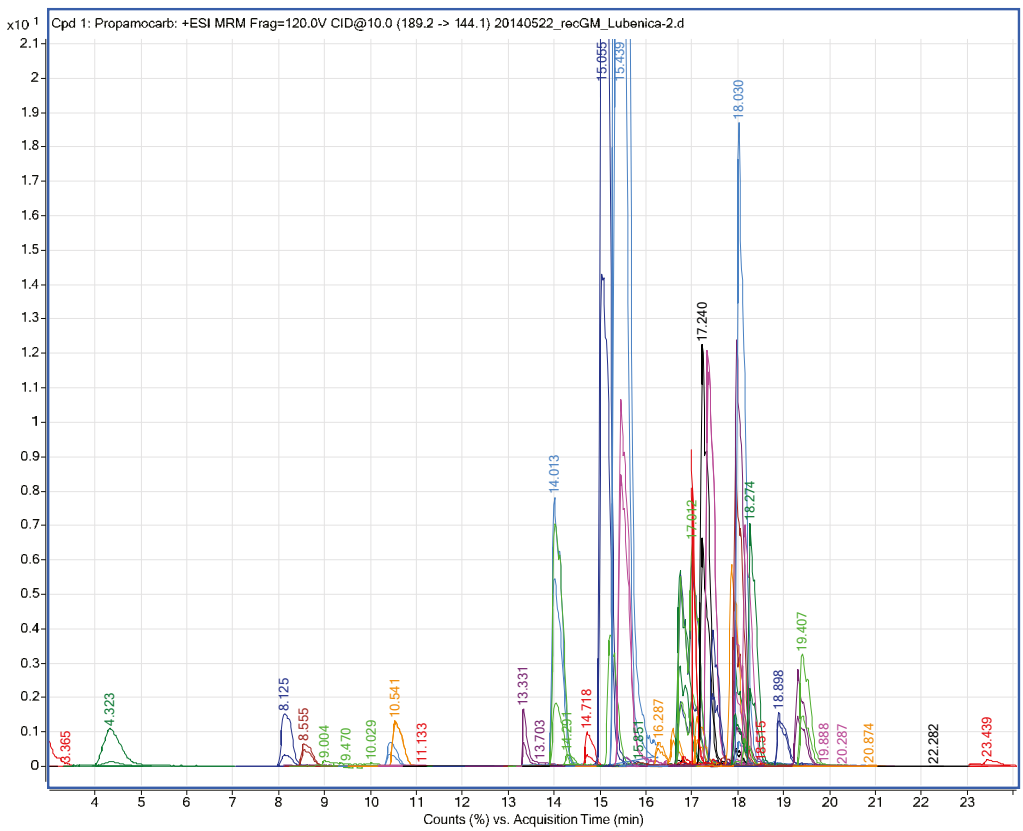

Figure 2. Overlaid MRM chromatograms of 55 pesticides standard in watermelon sample spiked at 10.0 ng/mL

RESULTS

The LC-MS/MS was used for the simultaneous residue determionation of 55 pesticides (acetamiprid, azoxystrobin, bupirimate, carbendazim, carbofuran, carbosulfan, chlorpyrifos, clothianidin, cyproconazole 1&2, cyprodinil, clethodim, difenconazol, dimethomorph, endosulfan alpha, epoxiconazole, fenhexamide, fenoxycarb, fenpropathrin, enpropimorph, fenvalerate, flusilazole, flutriafol, hexaconazol, imidacloprid, indoxacarb, krezoxym-methyl, metalaxyl-M, metconazol, methomyl, methoxyfenozide, methydathion, myclobutanil, oxadixyl, penconazol, pencycuron, pirimicarb, pirimifos-methyl, propamocarb, propoxur, propyconazol, pyraclostrobin, pyrimethanil, pyriproxifen, spiroxamine, tebuconazol, tebufenpyrad, tefluthrin, thiabendazole, thiacloprid, thiodicarb, triadimefon, triadimenol, trifloxystrobin, trifluralin and zoxamide) in the watermelon. Most of the studied pesticides are comprised by the monitoring programme of Serbia, regarding the substances whose presence and residue levels are studied in the food of plant origin (Off. Gazzet RS 58/2014). The active substances which were analyzed i.e. added to the list are bupirimate, clethodim and propamocarb as they are registered in the application with watermelons (Sekulić and Jeličić, 2011). The extraction was done using QuEChERS extraction kits with pre-weighed anhydrous salts in sealed packets which make it possible to add salts after adding organic solvents to samples, and to avoid an exothermic reaction that can compromise analyte recovery. Dispersive kits with sorbents and salts supplied in 15 mL centrifuge tubes accommodate the aliquot volumes specified by current AOAC and EN methodologies. These dispersive kits provide excellent recoveries and reproducibility for all types of fruits and vegetables.

The calibration was carried out in the watermelon matrix in order to overcome the matrix effect. The R^2 were >0.99 for all the studied pesticides ranging from 0.01 to 0.25 mg/mL. The obtained mean values of the responses were in the range from 61.0 to 114.2% with RSD <20.00%. The LOQs were 0.01 mg/kg (Bursić et al., 2014).

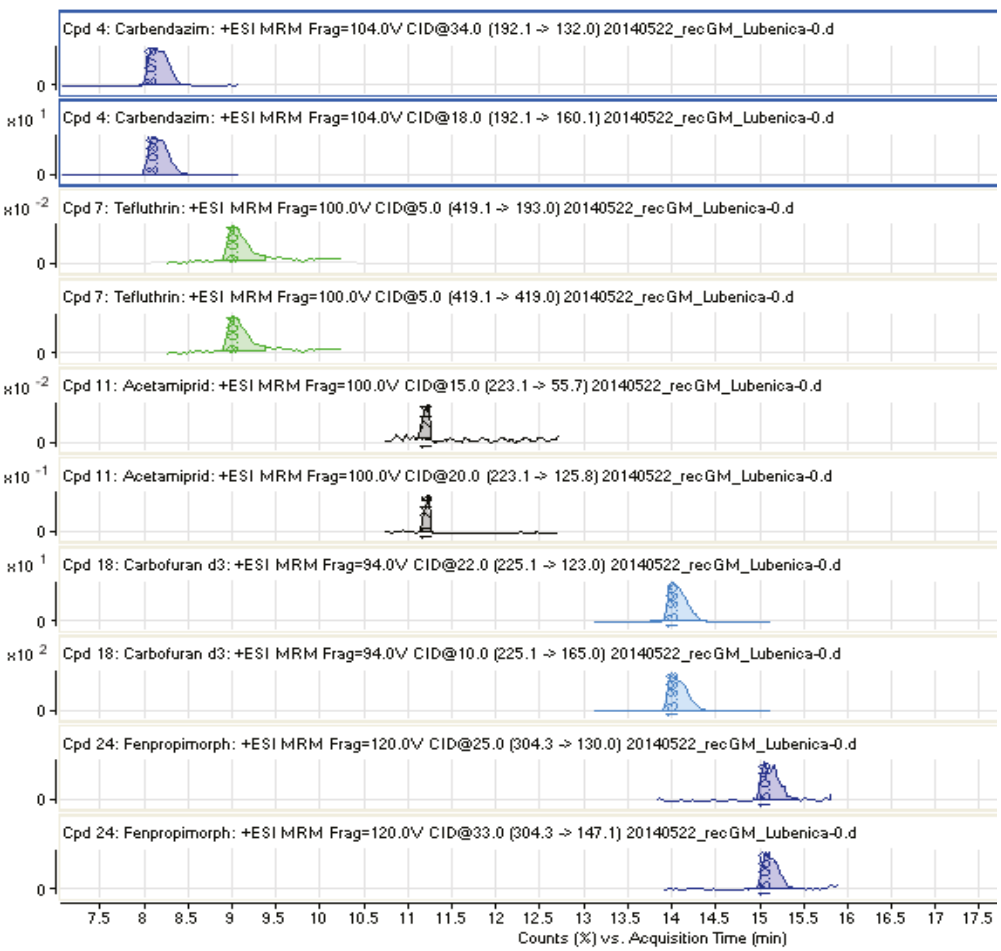

Figure 3. Pesticide residues detections in watermelon sample

DISCUSSION

The validated method which uses a liquid chromatography tandem mass spectrometry provides a very high sensitivity, good reproducibility, appropriate linearity and can be applied with the high reliability to the analysis of investigated pesticide residues in watermelon samples. The LOQs of 0.01 mg/kg confirm that the method is appropriate for the determination of pesticide residues in watermelon samples according to the regulations of the Serbian and EU MRLs.

The multiple detections were confirmed in the analysed samples. The most frequently detected pesticides were carbendazim, acetamiprid, tefluthrin and fenpropimorph. In only one sample the concentrations of carbendazim (0.134 mg/kg) and tefluthrin (0.304 mg/kg) were above the MRLs. The MRLs for this pesticide are 0.1 and 0.02 mg/kg, respectively. In the same sample the acetamiprid (0.005 mg/kg) and fenpropimorph (0.004 mg/kg) were detected, in the concentrations, which are much below the MRLs.

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DETERMINATION OF PHENOLIC COMPOUNDS IN PLANT EXTRACTS BY HPLC-DAD

Vojislava Bursić [1], Sonja Gvozdenac[1], Snežana Tanasković[2], Maja Meseldžija[1], Gorica Vuković[3], Boško Dedić[4] and Dejan Prvulović[1]

[1]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, Novi Sad, Serbia
[2] Faculty of Agronomy, University of Kragujevac, Cara Dušana 34, Čačak, Serbia
[3]Institute of Public Health of Belgrade, Bulevar despota Stefana 154a, Belgrade, Serbia
[4]Institute of Field and Vegetable Crops, Maksima Gorkog 30, Novi Sad, Serbia
E-mail: bursicv@polj.uns.ac.rs

ABSTRACT

Plants have the ability to synthesize secondary metabolites, biologically active substances involved in defense mechanisms against insects, pathogenic fungy and bacteria. Biochemical bases of their activity are related to the presence of specific molecules, among others, phenolic compounds. The aim of this study was to develop the validated method for the determination and quantification of phenolic acids, kaempferol and quercetin in ethanol leaf extracts of *Morus alba* L. and *Halascya sendtneri* (Boiss.) and leaf and bark extract of *Ailanthus altissima* (Mill.). The separation, quantification and validation of the individual phenols were performed by high performance liquid chromatography with diode-array detection (HPLC-DAD). The HPLC-DAD separation was achieved using a ZORBAX SB-Aq (5 µm particle size: 4.6 x 250 mm, Agilent). The mobile phase was acetonitrile with 2.0% acetic acid and Milli-Q water with 2.0% acetic acid in gradient mode, with the flow rate 1.0 ml/min. The obtained LOQs for all investigated phenolic acids were 0.03 µg/ml. The precision values, expressed as relative standard deviation (RSD, %), were lower than 10.19%. The developed HPLC-DAD chromatographic procedure exhibits linearity (R^2>0.99) for the concentrations from 10.0 to 100.0 µg/ml with the repeatability RSD less than 12.00%. An efficient, sensitive and reliable method is developed which can be applied in the analysis of real plant material samples to ferulic, trans-cinnamic, 2-hydroxy cinamic, gallic, caffeic, p-coumaric and chlorogenic acid, kaempferol and quercetin.

Key words: phenolic compounds, plant extracts, HPLC-DAD

INTRODUCTION

Current limitations in chemical pest control methods specify the scope for identifying safe, non-polluting rational methods in the control of economically important agricultural pests. Plants have developed many chemical defense mechanisms against insects in the evolution process and the ability to synthesize a broad range of different volatile and non-volatile chemical compounds called secondary metabolites (Wink 1993; Howe and Jander 2008), such as alkaloids, polyphenols, terpenoids, steroids, essential oils, lignans, sugars, and fatty acids, (Regnault-Roger et al. 2004; Isman 2006, Erturk et al., 2006; Shields et al., 2006; Koul, 2008). Naturally occurring plant compounds can affect the physiology or modify behavior of insect herbivores in terms of feeding and these are potentially suitable for use in integrated pest management (Schmutter, 1992).

The role of phenols in chemoecology especially on feeding behavior of herbivorous insects has been recognized since 1959, when Fraencke described phenolic compounds as "trigger" substances which induce or prevent the uptake of nutrients by animal herbivores. Plant polyphenols are secondary metabolites that constitute one of the most common and widespread groups of natural products (Cheynier et al., 2012). According to Bandaranayake (2002) these plant compounds have toxicological, pharmacological and ecological importance. Harborne (2002) emphasized that they have been implicated in diverse functional roles, including plant resistance against microbial pathogens and animal herbivores (insects), protection against solar radiation, besides reproduction, nutrition and growth.

High-performance liquid chromatography with diode array detection method (HPLC-DAD) is a widely used method for the analyses of phenolic compounds (Irakli et al., 2012; Andrejev and Bursić, 2013; Zhang et al., 2013; Bursić et al., 2013, Šućur et al., 2014). Although, in a recent literature data the liquid chromatography with tandem mass spectrometry (LC–MS/MS) systems is use for the determination of these substances (Gomez-Caravaca et al., 2013; Bursić et al., 2014).

The aim of this research was to develop and validate the HPLC-DAD method for the detection of phenolic compounds, such as ferulic, trans-cinnamic, 2-hydroxy cinamic, gallic, caffeic, p-coumaric and chlorogenic acid, quercetin and kaempferol in plant extracts. The method was evaluated in terms of linearity (R^2), reproducibility, limits of detection (LODs) and limits of quantification (LOQs).

MATERIALS AND METHODS

Chemicals and apparatus

All solvents used were of chromatography grade and were obtained from J.T. Baker (Deventer, Netherlands). The analytical standards manufactured by Sigma-Aldrich, which were used in the research work are ferulic acid (99.0%), trans-cinnamic acid (99.0%), 2-hydroxy cinamic acid (97.0%), gallic acid (99.9%), caffeic acid (98.0%), p-coumaric acid (98.0%), chlorogenic acid (95.0%), quercetin (98.0%), kaempferol (97.0%). The stock standard solutions were prepared by dissolving an analytical standard in methanol while the working solution i.e. the mixture of the studied phenol compounds was obtained by mixing and diluting the stock standards with mobile phase resulting in the final mass concentration of 100 mg/ml. The composite mixtures of all phenol compounds at appropriate concentrations were used to spike samples in validation data settings. Acetic acid was of "pure for analysis" grade by Carl Roth.

HPLC analysis

The chromatographic separation for phenolic compounds was achieved using the Agilent 1100 (Agilent Technologies, USA) HPLC system with a binary pump and diode array detector - DAD. The phenolic acids were separated on a ZORBAX SB-Aq (5 μm particle size: 4.6 x 250 mm, Agilent) column. The extracts were filtered through 0.45-μm syringe filters and directly injected through a 30 μl fixed loop into the column.

The mobile phase was acetonitrile with 2.0% acetic acid (solvent A) and Milli-Q water with 2.0% acetic acid (solvent B) in gradient mode, with the flow rate of 1.0 ml/min. This was equipped with a ZORBAX SB-Aq column. The gradient was as follows: 92% A at 0 min, 80% A at 18 min, 60% A at 25 min, 55% A at 30 min, 35% A at 40 min and 20% A at 42 min. Stop time was 2.5 minutes.

Validation parameters

The detector linearity response was checked by preparing the blank plant extract sample (leaves and root, separately) according to the Generalić et al. (2012) method and after the extraction the residue was diluted in 1.5 ml of the phenol compounds mixture standard in mass concentrations of 10.0, 25.0, 50.0 and 100.0 μg/ml.

The extract preparation

Plant material (10.0 g) was extracted with 70% ethanol (100.0 ml) as a solvent. Ethanol extracts of *Morus alba* L. and *Halascya sendtneri* (Boiss.) leaves and *Ailanthus altissima* (Mill.) leaves and bark were used in the analysis. The extracts were filtered through 0.45 μm syringe filters and directly injected into the HPLC-DAD. The repeatability of the method was determined by analyzing the sample of the same mass concentration level (10.0 μg/ml) in six replicates and shown through the relative standard deviation - RSD (%). The detection limit (LOD) was defined as the amount of phenolic compounds which produces the signal three times the noise signal. The quantification limit (LOQ) is the amount of phenolic compounds produces a signal ten times the noise signal. The LODs were determined by adding 100 ml of phenol compounds mixture standard to the concentration of 1.0 mg/ml, in 0.5 g of the sample in six replicates and the LOQs was calculated.

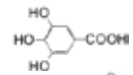
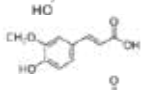
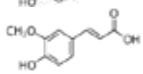
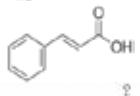
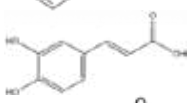
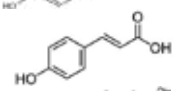
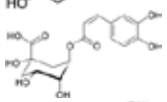
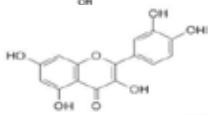
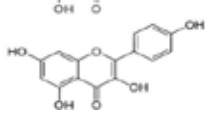
RESULTS

The method was evaluated in terms of linearity and repeatability, LOD and LOQ for ferulic, trans-cinnamic, 2-hydroxy cinamic, gallic, caffeic, p-coumaric and chlorogenic acid, quercetin and kaempferol.

HPLC-DAD chromatogram of standard solutions of the phenolic compounds preparing in mobile phase was shown in Figure 1.

Some of the validation parameters are shown in Table 1. The obtained LODs for all investigated phenolic compounds were 0.01 μg/ml with the LOQs of 0.03 μg/ml.

Table 1. R^2, repeatability (RSD, %) and structural formula investigated phenolic compounds

Phenolic acid	Structural formula	Retention time (min)	R^2	Repeatability (RSD, %)
Gallic acid		4.58	0.9998	6.76
Ferulic acid		22.48	0.9966	2.42
2 hydroxy cinnamic acid		24.00	0.9967	9.61
Trans cinnamic acid		26.43	0.9949	5.09
Caffeic acid		13.29	0.9984	9.48
p-Coumaric acid		18.94	0.9985	2.74
Chlorogenic acid		11.21	0.9996	4.18
Quercetin		40.75	0.9992	7.25
Kaempferol		37.67	0.9996	11.93

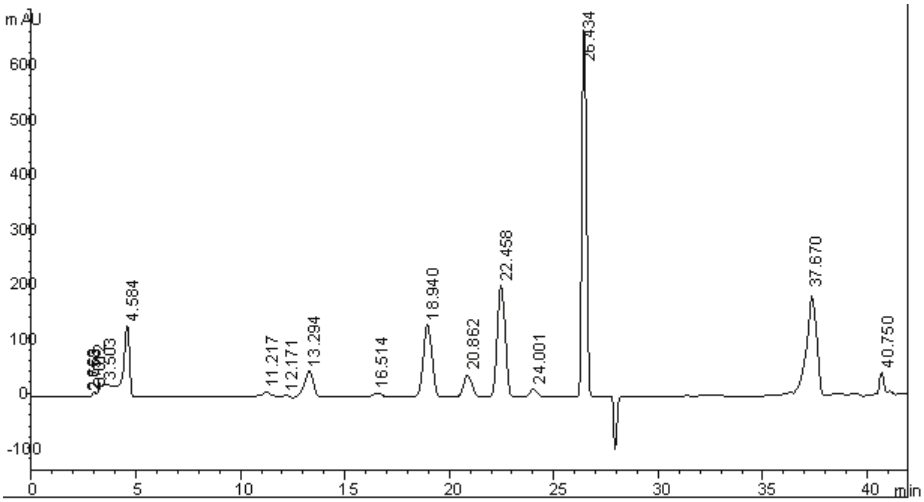


Figure 1. HPLC-DAD chromatogram of phenol compounds

Some of the calibration curves (gallic acid, caffeic acid, 2-hidroxycinnamic acid and kaempferol) were shown in Figure 2.

DISCUSSION

Phenolic compounds are found throughout the plant kingdom but the type of compounds present varies significantly depending on the taxonomic affiliation (Lattanzio, 2006). Extensive studies have been carried out to identify plant phenols with insecticidal properties and in *in vitro* experiments of Wójcicka (2010), phenolic compounds exerted negative influence on the feeding, reproduction, growth and survival of the *Sitobion avene, Schizaphis gramini* and *Mysus persicae*. Based on the large number of literature data, the most commonly found phenolic acids, with proved insecticidal and/or antifeedant activity, in plant extracts are quercetin and kaempferol, gallic, feruic and caffeic acids (Patton et al., 2005; Anonymous 1, 2006; Mesbah et al., 2007; Pavela, 2011; Zhang et al., 2013; Ladhari et al., 2013). Quercetin is one of the most abundant flavonoids and the defense secondary metabolite in plants. Liu-Shou-Zhu et al. (2007) suggest that quercetin at low concentrations can cause immune response of *Tenebrio molitor*. According to Mesbah et al. (2007), quercetin extracted from sunflower plants caused abnormal behavior, namely feeding arrest, growth inhibition and development retardation of *Spodoptera littoralis* larvae, deformation of pupae, moths and reduction up to 50% of laid eggs. Ismail et al. (2005) also described the antifeeding effects of well known flavonol glycosides, such as quercetin-3-O-glucopyranosides and kaempferol, while Mitchell et al. (1993) presented results indicating that among others, kaempferol and quercetin inhibit ecdysone 20-monooxygenase activity of *Aedes aegypti* adult females, wandering stage larvae of *Drosophila melanogaster* and pre-wandering and wandering stage of *Manduca sexta* larvae. All this implicated that mentioned compounds may function as biopesticides affecting insect ecdysteroidogenesis. It has become a common practice in USA to use C8-C12 alkyl ester of gallic acid to protect wooden material from insects feeding (Anonymous, 2006). However, according to Patton et al. (2005) caffeic acid expressed antifeeding activity towards adult Japanese beetles (*Popillia japonica*). Singh at al. (2014) represent that tomatoes are rich source of secondary metabolites, and its hairy roots efficiently produce phenolic compounds, such as rutin,

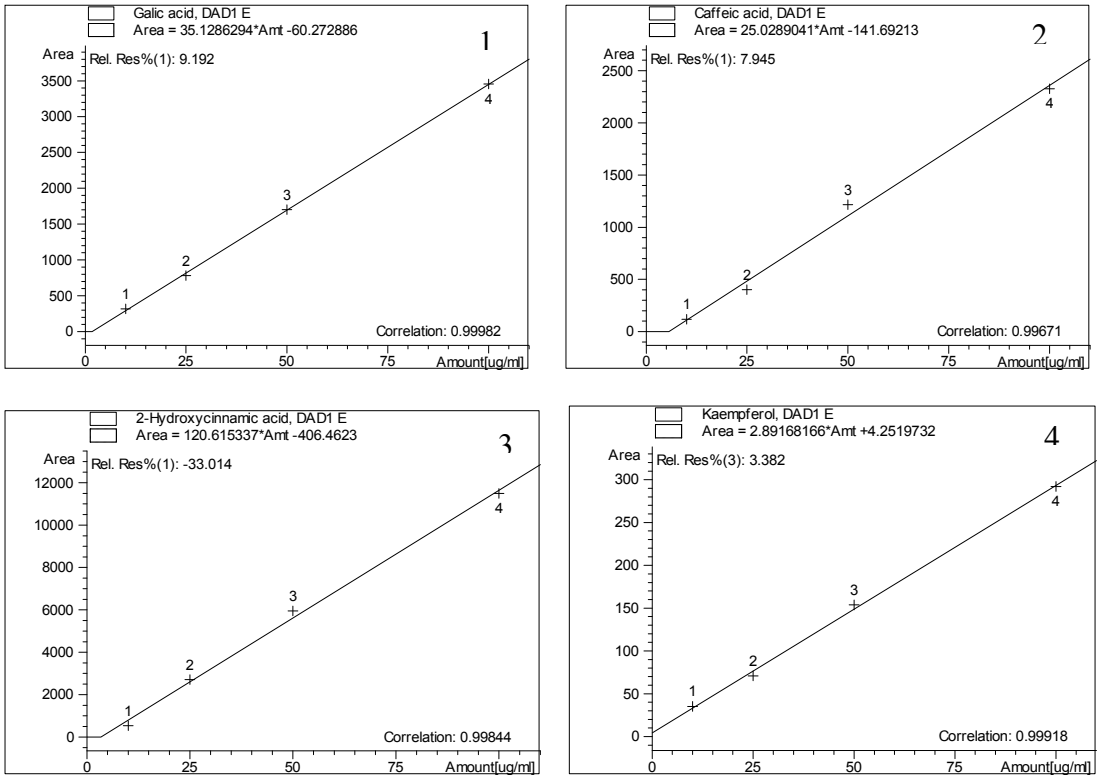


Figure 2. Calibration curves (1-gallic acid, 2-caffeic acid, 3-2-hidroxycinnamic acid, 4- kaempferol)

quercetin, kaempferol, gallic acid, protocatechuic acid, ferulic acid, chlorogenic acid, and caffeic acid. At 100 μL/g concentration, the phenolic compounds caused 53.34 and 40.00% mortality of *Helicoverpa armigera* and *Spodoptera litura*, respectively, after 6 days, while surviving larvae of both species, after 6 days showed 85.43 and 86.90% growth retardation, respectively. Also, chlorgenic acid, detected in extracts used in this work, has been described as an antifeedant and digestibility reducer in aphids (Miles and Oertli, 1993).

- In order to estimate the biochemical basis of plant extracts an efficient, sensitive and reliable HPLC-DAD method was validated. It can be applied to ferulic, trans-cinnamic, 2-hydroxy cinamic, gallic, caffeic, p-coumaric and chlorogenic acid and quercetin and kaempferol in the analysis of real plant extract samples.
- The obtained LODs for all investigated phenolic compounds were 0.01 μg/ml with the LOQs of 0.03 μg/ml.
- The correlation coefficient of all phenolic compounds were R^2>0.99 in the concentration range from 10.0 to 100.0 μg/ml with the repeatability RSD less than 11.93%.

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PHOTOCHEMICAL PROCESSES AND THEIR USE IN REMEDIATION OF WATER CONTAINING PESTICIDES

Anđelka Tomašević and Slavica Gašić

Institute of Pesticides and Environmental Protection, Banatska 31b,
11080 Belgrade-Zemun, Serbia;
andjelka.tomasevic@pesting.org.rs; slavica.gasic@pesting.org.rs

ABSTRACT

The most pesticides are resistant to chemical and natural photochemical degradation and the development of adequate methods for remediation of contaminated waters is very important. There are different methods of removing pesticide residues from water, but we would discuss photodegradation methods. According to literature data, the most beneficial photochemical processes for removal of pesticide residues from water are two famous Advanced Oxidation Processes heterogeneous photocatalysis with semiconductor oxides TiO_2 and ZnO, and homogeneous photo-Fenton treatment, as well as direct UV photolysis. The photochemical investigations may contribute toward a better understanding of pesticide behavior in the environment: more information on the degradation time of active ingredients, on their activity and environmental fate can be obtained by studying the kinetics of any photochemical reaction.

Key words: pesticides, water treatment, photodegradation, photocatalysis, photolysis

INTRODUCTION

A large number of pesticide active ingredients have been registered and marketed for pest control purposes around the world. Pesticide residues are widespread in streams and shallow groundwater, and their occurrence follows the patterns of geographic and seasonal use of pesticides in any area. It is known that high concentrations of herbicides are often found in the areas with extensive agricultural activity, whereas many insecticides are found in urban streams. Agricultural and urban runoffs, leaching from pesticide waters, industrial-scale pest control operations, direct application of pesticides to control aquatic insects and vegetation, and domestic usage are possible causes of pesticide contamination in drinking water sources.

Removing pesticide residues from water are very difficult and pesticide pollution in surface and groundwater has been recognized for many years as an important issue in a number of countries.

There are two types of pesticides contaminated aqueous media: wastewaters from pesticide manufacturing plants, agricultural fields, and equipment rinsing operations (rinse water or rinsate), as well as surface water and groundwater. Whereas wastewaters often contain very high level (milligram per liter or more) of pesticides, surface water and groundwater usually contain only trace amounts of pesticides (microgram per liter or less), but these often occur as a more complex mixture (Kolpin et al., 1998).

Various processes have been investigated to reduce pesticide concentrations in water, and to minimize the potential health risks. Conventional wastewater treatments involve mechanical, biological, physical and chemical processes. These methods of water disinfection and decontamination can solve many of the problems. However, these methods are often chemically, energetically and operationally intensive, focused on large systems, and thus require considerable infusion of capital, engineering expertise and infrastructure,

all of which precludes their use in much of the world. Furthermore, intensive chemical treatments, involving ammonia, chlorine compounds, hydrochloric acid, sodium hydroxide, ozone, permanganate, aluminum and ferric salts, coagulation and filtration aids, anti-scalants, corrosion control chemicals, and ion exchange resins can add to the problems of contamination and salting of freshwater sources. Physical treatments as coagulation, flocculation, sedimentation, flotation, filtration, adsorption etc., transfer the pollutants from the liquid phase to a new phase instead for their elimination. Chemical treatments can also release to the air or in "purified" water more hazardous materials than originally was located in the contaminated one (Malato et al., 2009).

Photodegradation is degradation of a photodegradable molecule caused by the absorption of photons, particularly those wavelengths found in sunlight, such as UV, VIS or IR light. Photodegradation includes photodissociation, the breakup of molecules into smaller pieces by photons. It also includes the change of molecules shape to make it irreversibly altered, such as the denaturing of proteins, and the addition of other atoms or molecules. A common photodegradation reaction is oxidation and this is used by some drinking water and wastewater facilities to destroy pollutants. Photochemical processes could be used for treatment of many hazardous organic contaminants in water, aqueous waste streams, soils and groundwater, and for decontamination of water from pathogens.

The objective of this work is to present several technologies based on photochemical processes for the near ambient degradation of pesticides from water and wastewaters. The most beneficial processes for removal of pesticide residues from water are direct UV photolysis and Advanced Oxidation Processes (AOPs).

PHOTOLYSIS

Photolysis (direct photodegradation reaction) is photodegradation process without any catalysts and use light only for degradation of different organic molecules, including pesticides and related compounds. Direct irradiation will lead to the promotion of the pesticides to their excited singlet states and such excited states can then undergo among homolysis, heterolysis or photoionization processes. The photolysis taking place in the various aqueous and non-aqueous medium, such as water, different aqueous solutions and medium, different organic solvents, and the other medium (water/soil suspension, thin films, glass, etc) (Burrows et al., 2002). Photolysis by solar light is limited and various lamps have been used for irradiation of contaminated water solution (mercury, xenon, fluorescent, mercury-xenon, etc.). The photolysis of pesticides in aqueos solution depends on the different reaction parameters such as type of light, lamp distance, temperature, initial concentration of pesticides, type of water, pH, the presence of humic and fulvic acids, the presence of O_2, O_3, O_2/O_3 and H_2O_2, the presence of inorganic ions and organic matter dissolved in water, etc (Burrows et al., 2002; Tomaševic at al., 2010a; Tomašević, 2011; Tomašević & Gašić, 2012a; Tomašević & Gašić, 2012b).

ADVANCED OXIDATION PROCESSES

AOPs are near ambient temperature and pressure water treatment processes which involve the generation of hydroxyl radicals in sufficient quantity to effective water purification. Hydroxyl radical •OH is a powerful, non selective oxidant, which reacts rapidly with most organic compounds as pesticides, halogenated hydrocarbons, aromatic compounds, phenol-compounds, detergents, etc (Legrini et al., 1993). AOPs include catalytic and photochemical methods which use H_2O_2, O_3 or O_2 as the oxidant. The main advantage of these processes is a complete mineralization of many organic pollutants to water, CO_2, mineral salts, and non-toxic compounds (Andreozzi et al., 1999; Neyens & Baeyens, 2003). AOPs involve different homogeneous and heterogeneous photocatalytical processes. Homogeneous photocatalytic oxidation employs various oxidation system (H_2O_2, O_3, Fenton reagent, etc.) either alone or in combination with UV, visible, and natural solar light (Legrini et al., 1993). Heterogeneous photocatalytical processes, named heterogeneous photocatalysis are combination of UV or solar light, various catalysts (TiO_2, ZnO, ZnS, ZrO_2, CdS, SnO_2, WO_3, etc) and different oxidants (H_2O_2, $K_2S_2O_8$, KIO_4, $KBrO_3$, etc.) (Andreozzi et al., 1999; Daneshvar et al., 2003; Legrini et al., 1993; Tomašević et al., 2014).

Heterogeneous photocatalysis

Heterogeneous photocatalysis applies inexpensive semiconductors (TiO_2, ZnO, etc.) to mineralize even very stable organic compounds. Photocatalytic reactions occur when charges separation is induced in a large band gap semiconductor (TiO_2, $h\nu \geq E_g = 3.2$ eV). The absorption of light by the photocatalyst greater than its band gap energy (for TiO_2 $\lambda < 390$ nm) excites an electron from the valence band of the irradiated particle

to its conduction band, producing a positively charged hole, h^+ in the valence band and an electron, e^- in the conduction band. The hole, h^+ in the valence band may react with water absorbed on the surface to form hydroxyl radicals, and on the other hand, the conduction band electron, e^-, can reduce absorbed oxygen to form peroxide radical anions, that can further disproportionate to form •OH radical through various pathways. The •OH radicals react with contaminants via oxidative processes, and the band electron may also react directly with the contaminants via reductive processes. The main equations of the heterogeneous photocatalysis are (Andreozzi et al., 1999; Legrini et al., 1993; Daneshvar et al., 2003):

$$C + h\nu \rightarrow C\,(e^- + h^+) \qquad (1)$$

$$h^+ + H_2O \rightarrow \bullet OH + H^+ \qquad (2)$$

$$e^- + O_2 \rightarrow O_2\bullet^- \qquad (3)$$

Among AOPs, heterogeneous photocatalysis in the presence of TiO_2 appears as an effectively destructive reaction (Malato et al., 2002a, 2002b; Tomašević et al., 2010a; Tomašević, 2011). Titanium dioxide is the most common semiconductor used in photocatalytical processes because of its high catalytical activity, non-toxicity, chemical and biologycal stability, water insolubility, etc. (Malato et al., 2002b; Tomašević, 2011). Also, it does not involve mass transfer, it can be carried out under ambient conditions (atmospheric oxygen is used as oxidant), has strong resistance to chemical breakdown and photocorrosion, and may lead to complete mineralization of organic carbon into CO_2 (Malato et al., 2009). Among the various semiconductors employed, TiO_2 photocatalytic reaction is receiving increasing attention because of its low cost when using sunlight as the source of irradiation (Malato et al., 2009).

Zinc oxide is also frequently used as a catalyst in heterogeneous photocatalytic processes and has good characteristics on degradation and mineralization of pesticides and other organic pollutants (Behnajady et al., 2006; Tomašević et al., 2010a; Tomašević, 2011; Tomašević et al., 2014). The biggest advantage of ZnO in comparison to TiO_2 is that it absorbs over a larger fraction of the UV spectrum and according the literature data the corresponding threshold wavelength of ZnO is 440 nm (Malato et al., 2009), relatively to 425 nm (Behnajady et al., 2006).

The rate and efficiency of a heterogeneous photocatalytic reaction depends on a number of factors, including initial concentration of reactant, initial concentration of catalyst, pH, light intensity, temperature, concentration of oxygen, presence of scavengers and various ions, etc (Malato et al., 2009; Tomašević et al., 2010a; Tomašević, 2011; Tomašević et al., 2014).

Photo-Fenton processes

The reactions discovered in 1894 and have been called Fenton's reactions using iron catalyzed hydrogen peroxide and belong to AOPs. The classic Fenton's reagent is a mixture of ferrous ion and H_2O_2 in acidic solution or suspension (Neyens & Baeyens, 2003; Tamimi et al, 2008):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH \qquad (4)$$

Equation (4) presents the most important steps of a Fenton reaction and involves electron transfer between H_2O_2 and Fe^{2+} and the resulting production of highly reactive hydroxyl radical •OH and potentially reactive Fe^{3+} species. The degradation of pesticides by Fenton's reagent can be strongly accelerated under UV or various types of visible light. This process is the photo-Fenton reaction (Malato et al., 2002a, 2002b; Tamimi et al, 2008; Tomašević et al., 2010b). Equation (4) is the key of photo-Fenton processes. The obtained Fe^{3+} ion or its $Fe(OH)^{2+}$ complexes act as light absorbing species, that produce another hydroxyl radical, while the initial Fe^{2+} ion is regained (equation 5):

$$Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + \bullet OH \qquad (5)$$

The main advantage of the photo-Fenton process is light sensitivity up to a wavelength of 600 nm (Malato et al., 2002a, 2002b). The kinetics of a photo-Fenton reaction depends on a different factors, such as initial concentration of reactant and its characteristics, initial iron concentration and iron source, initial concentration of H_2O_2, pH, light intensity, and temperature (Tamimi et al, 2008; Tomašević at al., 2010b; Tomašević, 2011).

PRACTICAL APPLICATION OF PHOTOCHEMICAL PROCESSES

Photocatalysis should be a suitable technology as the final stage of purification of biologically or physically pretreated wastewater, in particular in sun-rich areas. AOPs have become important hazardous water pollution treatment techniques, with an increasing number of technically and economically feasible applications (Malato et al., 2002a; Kiss et al., 2011). The main reason

for the use of AOPs is usually severe water pollution and/or toxicity which cannot be treated in bioreactors. The first outdoor engineering-scale reactor developed was a converted solar thermal parabolic-trough collector in which the absorber/glazing-tube combination had been replaced by a simple Pyrex glass tube through which contaminated water could flow. Since that time, research all over the world has advanced a number of reactor concepts and designs, including concentrating and non-concentrating reactors (Malato et al., 2002a).

Solar photocatalytic treatment plants are usually operated in batch mode. Polluted water must first be pre-treated so the organic destruction process takes place under the best possible conditions. After this, the catalyst is added and the mixture is pumped in batches through the chemical reactor (solar collector field) until the pollutants are degraded. Depending on the nature of the contaminants to be treated, some potentially useful chemical oxidants can be added to enhance process efficiency. When the process is complete, post-treatment processes must adjust the water chemistry to conditions suitable for discharge (Malato et al., 2002a).

The water from washing the pesticide bottles is treated in batches until 80% of the TOC has been mineralized. At this point, the water is transferred to the post-treatment (iron precipitation, sedimentation and recuperation), and either reused for bottle washing or discharged for irrigation through an activated carbon filter to ensure discharge quality. The water for reuse is pumped back to wash the shredded plastic until TOC is 100 mg L^{-1}. In this closed cycle, water may be reused up to about 10 times before final discharge. About 95% of the contaminants are mineralized and a granulated activated carbon filter removes the remaining 5% (Blanco et al., 2004).

Water disinfection is also an important field of solar catalytic reactor application. The rate of bacterial decontamination by solar radiation is proportional to radiation intensity and temperature, and inversely proportional to the depth of the water, due to light dispersion (Malato et al., 2009). The amount of radiation attenuated by this depends on the wavelength range. For example, between 200 and 400 nm, the reduction is less than 5% m^{-1} depth, and at longer wavelengths it may be up to 40% m^{-1}. The most destructive wavelengths for microbial life are in the near UV-A spectrum (320–400 nm), whereas the spectral band from 400 to 490 nm is the least harmful. Likewise, differences in bacterial inactivation rates at temperatures between 12 and 40 °C are negligible, but the bactericidal action is accelerated twofold when the temperature rises to 50 °C, probably due to the synergetic effect between radiation and temperature (Kiss et al., 2011).

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REMOVAL OF CARBAMATE RESIDUES FROM WATER BY DIFFERENT PHOTOCHEMICAL PROCESSES

Anđelka Tomašević[1], Slavica Gašić[1], Dušan Mijin[2], Slobodan Petrović[2], Ana Dugandžić[2] and Olivera Glavaški[3]

[1]Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade-Zemun, Serbia;
andjelka.tomasevic@pesting.org.rs; slavica.gasic@pesting.org.rs
[2]University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11000 Belgrade, Serbia;
kavur@tmf.bg.ac.rs; sloba@tmf.bg.ac.rs; adugandzic@tmf.bg.ac.rs
[3]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia, olivera.glavaski@nsseme.com

ABSTRACT

The photodegradation of the carbamate insecticides methomyl and carbofuran in different types of water, in the presence/absence of TiO_2/ZnO, using several light sources were studied. The effect of several operational parameters to degradation kinetics was investigated. The optimal concentration of catalysts was found to be 2.0 g L^{-1}. On the base of the Langmuir-Hinshelwood mechanism, a pseudo first-order kinetic model was illustrated. The rate of photodecomposition of methomyl and carbofuran were measured using ultraviolet spectroscopy (UV) and high performance liquid chromatography (HPLC), while their mineralization were followed using ion chromatography (IC) and total organic carbon (TOC) analyzer. The results of investigation are shown that the photochemical removal of the pesticides is applicable method for the purification of water.

Keywords: methomyl, carbofuran, photodegradation, water remediation

INTRODUCTION

Methomyl (IUPAC name S-methyl N-(methylcarbamoyloxy)thioacetimidate is an insecticide/acaricide widely used in agriculture. It is used for control of a wide range of insects and spider mites in fruit, vines, olives, hops, vegetables, ornamentals, field crops, cucurbits, flax, cotton, tobacco, soya beans, etc. Also it can be used for control of flies in animal and poultry houses and dairies. Formulations types for this active ingredient are SL, SP, and WP. The current regulation status of this active ingredient in Annex 1 is approved, Reg. (EC) No 1107/2009, expiration of inclusion: 31/08/2019 (EU Pesticide Database, 2015; MacBean, 2012).

Carbofuran (IUPAC name 2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is systemic insecticide with predominantly contact and stomach action. It is used for control of soil-dwelling and foliar-feeding insects and nematodes in vegetables, ornamentals, beet, maize, sorghum, sunflowers, oilseed rape, potatoes, alfalfa, peanuts, soya beans, sugar cane, rice, cotton, coffee, cucurbits, tobacco, lavender, citrus, wines, strawberries, bananas, mushrooms and other crops. This active ingredient is prepared as FS, GR, SC and WP formulation. The current regulation status of this active ingredient in Annex 1 is not approved, Reg. (EC) No 1107/2009 (EU Pesticide Database, 2015; MacBean, 2012).

There are different methods of removing pesticides from water, but our focus was on the methods based on photodegradation. The photolysis (direct photodegradation reaction or photodegradation process without catalyst) use light only for degradation of different environmental contaminants, including pesticides residues (Burrows et al., 2002; Tomašević et al., 2010a). Advanced Oxidation Processes (AOPs) include catalytic and photochemical methods which use H_2O_2, O_3 or O_2 as the oxidant. The principal active species in these systems is the hydroxyl radical •OH, which is an extremely reactive and non-selective oxidant for organic contaminants (Legrini et al., 1993). The main advantage of these processes is a complete mineralization of many organic pollutants (Andreozzi et al., 1999). Heterogeneous photocatalysis is combination of semiconductor particles (TiO_2, ZnO, Fe_2O_3, CdS, and ZnS), UV/solar light and different oxidants (H_2O_2, $K_2S_2O_8$, KIO_4, and $KBrO_3$) (Andreozzi et al., 1999; Daneshvar et al., 2003; Legrini at al., 1993). Fenton's processes belong to AOPs and utilize H_2O_2 activation by iron salts. The classic Fenton's reagent is a mixture of ferrous ion and H_2O_2 in acidic solution or suspension. The degradation of pesticides by Fenton's reagent can be strongly accelerated upon UV or UV-visible light and this process is the photo-Fenton reaction (Malato et al., 2002a, 2002b; Tamimi et al, 2008; Tomašević et al., 2010b).

Methomyl and carbofuran have been photodegradated using photolysis and AOPs. Different catalysts have been used, mostly TiO_2 (Malato et al., 2002b; Tomašević et al., 2010a; Tomašević, 2011; Mahalakshmi et al., 2007), as well as photo-Fenton reaction (Malato et al., 2002b; Tamimi et al, 2008; Tomašević et al., 2010b; Tomašević, 2011; Li-An et al., 2011). The aim of the present work is to study the photolysis and photocatalytic degradation of insecticides methomyl and carbofuran in water using different light sources. The effect of parameters such as lamp distance, water type, temperature, initial concentration of catalyst, initial pesticides concentration, and pH were studied.

MATERIALS AND METHODS

All chemicals used in the investigation were of reagent grade and were used without further purification. Analytical standard of methomyl (99.8%) was received as a present from Du Pont de Nemours, USA. Analytical-grade carbofuran (99.2%) were granted by FMC, USA. The photodegradation of methomyl and carbofuran were studied by preparing solutions containing 16.22 mg L^{-1} of methomyl and 22.12 mg L^{-1} of carbofuran. Both, photodegradation and analytical procedures were reported elsewhere (Tomašević et al., 2010a; Tomašević et al., 2010b; Tomašević, 2011).

RESULTS AND DISCUSSION

Photolysis of methomyl and carbofuran

The photolysis of methomyl in different types of water (deionized, distilled and sea water) was performed (Tomašević et al., 2010a; Tomašević, 2011) and the influence

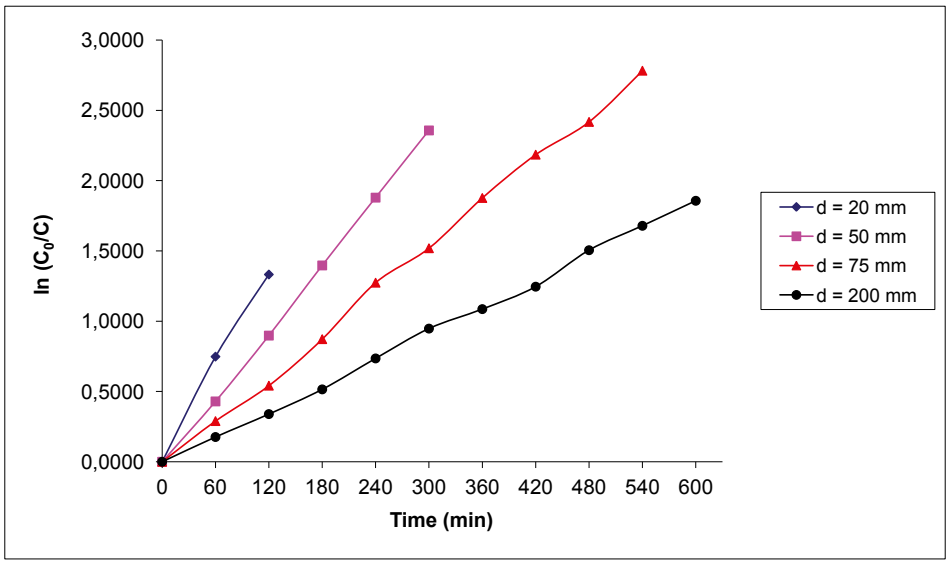

Figure 1. The effect of lamp distance on the photolysis rate of methomyl (λ = 254 nm, temperature = 20 ^{0}C, pH = 6.0).

of reaction parameters to degradation of pesticide were investigated. All the experiments were carried out under monochromatic ultraviolet light at 254 nm because light of 366 nm had no effect previously on direct methomyl photodegradation. The studies showed that the photolysis reactions depend on the lamp distance (Figure 1), water type, reaction temperature, and pH (Figure 2). The distance between the lamp and surface solution varied from 20 to 200 mm. The duration of exposure of methomyl solution to irradiation depended on the distance between the lamp and surface solution. The results of photolytic dissipation of methomyl in aqueous solution with increasing time of exposure to UV irradiation are shown in Figure 1. The reaction rate was 3.7 times higher (k, 0.0194 min^{-1}) when the lamp was placed at 20 mm distance from the reaction mixture surface, in comparision to the reaction rate (k, 0.0053 min^{-1}) when the lamp was placed 200 mm. The influence of water type was investigated by conducting photodegradation experiments of methomyl at 254 nm in deionized (pH 5.2), distilled (pH 5.5) and seawater (pH, 7.9: concentration of Cl^- ions, 26.2 g L^{-1}) and the experiments showed that the reaction rate was highest in distilled water and lowest in sea water. The photolysis rate of methomyl increase in the temperature range from 10 ^{0}C (k, 0.0076 min^{-1}) to 50 ^{0}C (k, 0.0098 min^{-1}), while half-lives decrease from 91.2036 min to 70.7293 min, respectively. The obtained results imply that the photodegradation rate of methomyl is highest in weak acidated (pH, 6.0) and lowest in strong acidated solution (pH, 3.15) (Figure 2).

In addition, our studies showed that both of monochromatic ultraviolet lights (254 nm and 366 nm) had no effect on direct carbofuran photodegradation.

Photocatalytic degradation of methomyl and carbofuran under 366 nm and 315-400 nm lights

In our experiments with methomyl and carbofuran we used two types of catalyst: the most popular semiconductor photocatalysts TiO_2 and ZnO. The experiments were performed with 0.5 g L^{-1}, 1 g L^{-1}, 2 g L^{-1}, and 3 g L^{-1} of either TiO_2 or ZnO. The most important advantage of ZnO over TiO_2 is that it absorbs over a larger fraction of the UV spectrum (Behnajady et al., 2006; Malato et al., 2009). The effect of parameters such as initial concentration of catalyst, initial methomyl concentration, and pH were studied.

For methomyl degradation the optimal concentration of the catalysts was found to be 2 g L^{-1}. The obtained results (Table 1) showed that photodegradation was much faster when ZnO was used than TiO_2. The results imply that the photodegradation rate was highest in acidic solution and lowest in alkaline solution. The ion chromatography (IC) results showed that mineralization led to the formation of sulfate, nitrate and ammonium ions during the process. Mineralization of sulfur atoms into sulfate ions was almost complete and the maximum expected value for sulfate ions (around 9.60 mg L^{-1}) was obtained. When TiO_2 was used as a catalyst, mineralization of organic carbon was incomplete

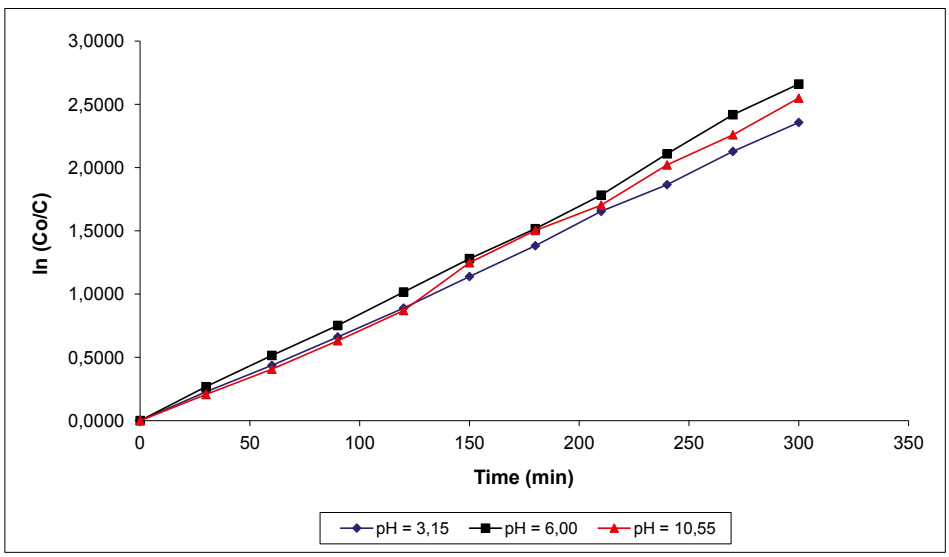

Figure 2. The effect of pH on the photolysis rate of methomyl (λ = 254 nm, lamp distance = 100 mm, temperature = 20 ^{0}C).

and about 80% of initial TOC disappeared after 8 hours (under 300 W Osram lamp, 315-400 nm) or after 6 hours (under 366 nm). These results are in accordance with those of other researchers (Oller et al., 2006). With ZnO, the same TOC disappearence occurred only after 4 hours.

Table 1. Kinetics of methomyl photodegradation at 366 nm

Methomyl	Parameters	Deionized water
with 2.0 g L^{-1} of TiO_2	k (min^{-1})	0.0058
	R	0.9880
	$t_{1/2}$ (min)	119.51
with 2.0 g L^{-1} of ZnO	k (min^{-1})	0.0120
	R	0.9915
	$t_{1/2}$ (min)	57.76

For carbofuran degradation the optimal catalyst was ZnO in concentration of 2 g L^{-1} (Figure 3). Also, in our previous study we investigated the influence of initial carbofuran concentration, and pH on the degradation rate (Tomašević, 2011). The increase of initial insecticide concentration conducted to decrease of carbofuran degradation rate and the photodegradation rate of carbofuran was higher in acidic than in alkaline conditions.

Degradation of methomyl and carbofuran under photo-Fenton process

Our experimental work was directing on catalytic wet peroxide oxidation of methomyl and carbofuran (photo-Fenton reaction). We used halogen lamp (576.6 nm) and two types of heterogeneous iron catalyst: Fe-ZSM-5 zeolite in concentration of 5 g L^{-1} and 1 g L^{-1}, and AlFe-pillared montmorillonite in concentrations of 5 g L^{-1}, 3 g L^{-1}, and 1 g L^{-1}. The effect of the catalyst concentration on photodegradation efficiency of methomyl is shown in Figure 4. With the AlFe-pillared clay catalyst, only 54% of methomyl was degraded after 5 hours of illumination. But, methomyl conversion with Fe-ZSM-5 zeolite was over 80% after 1 hour of reaction, and almost 100% of

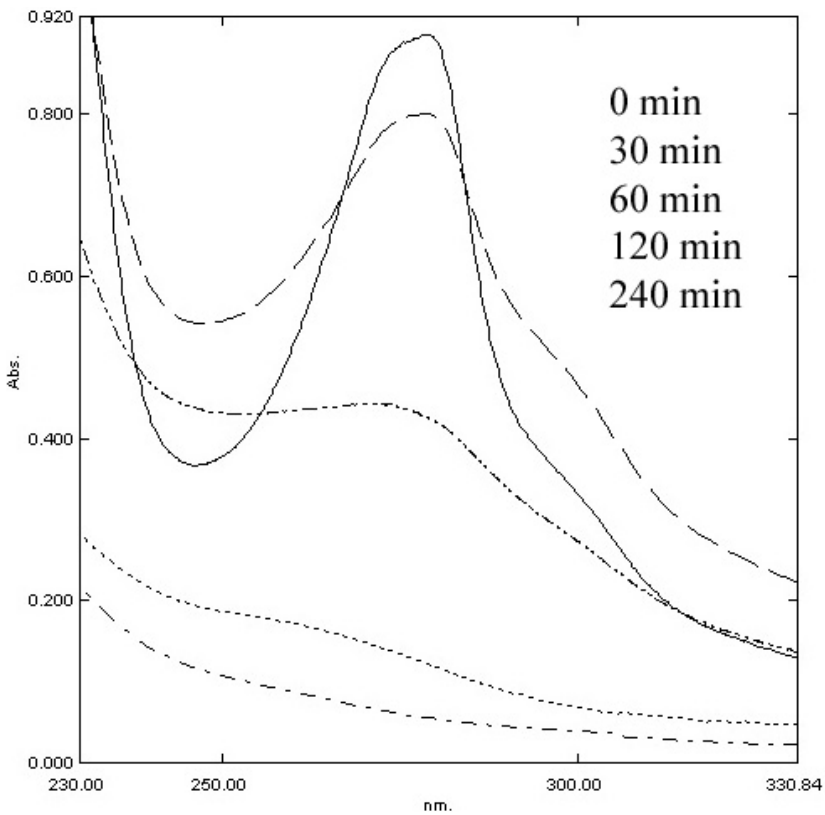

Figure 3. UV-VIS spectra changes of carbofuran (22.12 mg L^{-1}) in 2.0 g L^{-1} aqueous ZnO dispersion irradiated with an Osram Ultra vitalux lamp (315-400 nm).

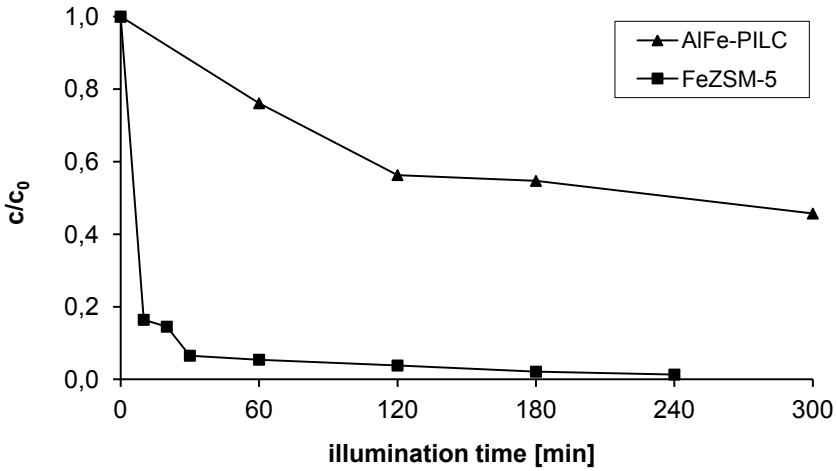

Figure 4. Photodegradation of methomyl with 5 g L^{-1} of catalysts (λ = 577.6 nm, temperature = 20 ^{0}C, pH = 3.5).

methomyl was degradated after 4 hours of irradiation. Both catalysts were shown to be active, but the zeolite-supported catalyst is better (Tomašević et al., 2007). Also, comparing the two catalysts, it is evident that the zeolite structure certainly stabilizes iron in a state favorable for catalytic process (Lázár et al., 2009). However, both applied catalysts showed no activity in the carbofuran conversion, even at an elevated temperature, 85 ^{0}C. The high chemical stability of carbofuran molecul could be explained by its electronic structure, but this claim requires further theoretical and experimental investigations. Also, we suppose that carbofuran can not enter clay channels with small openings, •OH positioned in the vicinity of Fe^{2+} stay unavailable to the reactant and reaction does not occur (Tomašević, 2011).

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APPLICATION OF PHOTOCHEMICAL PROCESSES FOR REMOVAL OF SULFONYLUREA AND CHLOROACETAMIDE RESIDUES FROM WATER

Anđelka Tomašević[1], Slavica Gašić[1], Dušan Mijin[2], Slobodan Petrović[2], Ana Dugandžić[2] and Olivera Glavaški[3]

[1]Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade-Zemun, Serbia;
andjelka.tomasevic@pesting.org.rs; slavica.gasic@pesting.org.rs
[2]University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11000 Belgrade, Serbia;
kavur@tmf.bg.ac.rs; sloba@tmf.bg.ac.rs; adugandzic@tmf.bg.ac.rs
[3]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia; olivera.glavaski@nsseme.com.

ABSTRACT

The photochemical degradation of the sulfonylurea and chloroacetamide herbicides nicosulfuron and dimethenamid-P in deionized water, in the presence of TiO_2, under polychromatic light (315-400 nm) was studied. The effect of several operational parameters to degradation kinetics was investigated. The optimal concentration of catalysts was found to be 4.0 g/L for nicosulfuron and 2.0 g/L for dimethenamid-P. During the process, the influence of operational parameters, such as the initial concentration of the catalyst (0.5-5 g/L), the initial pesticide concentration (5-50 mg/L), initial salt concentration (20-200 mM), and pH (1-12) was monitored. Reactions were followed by ultraviolet spectroscopy (UV), as well as high performance liquid chromatography (HPLC), ion chromatography (IC), and total organic carbon (TOC) analyzer.

Keywords: nicosulfuron, dimethenamid-P, photodegradation, operational parameters

INTRODUCTION

Nicosulfuron, 2-(4,6-dimethoxypyrimidin-2-ylcarbamoylsulfamoyl)-N,N-dimethylnicotinamide, is a selective systemic herbicide absorbed by the foliage and roots, with rapid translocation in xylem and phloem to the meristematic tissues.It has been widely used to protect corn, rice, citrus, vines, and potatoes. Despite the low persistence of nicosulfuron, its residues have been reported in many materials, such as soil, surface waters, and some crops. The low volatility and photodegradation, as well as long persistence of this herbicide under certain conditions have raised increasing concerns about the risk of contamination of surface and ground waters. Formulations types for this active ingredient are SL, SP, and WP (MacBean, 2012).

Dimethenamid-P, 2-chloro-N-(2,4-dimethyl-3-thienyl)-N-(2-methoxy-1-methylethyl) acetamide, belongs by its chemical properties and structure to the group of chloroacetamides and plays an important role in the crop protection of broadleaf weeds and annual grasses in row crops (Glavaški et al., 2014), primarily in corn, soybean, and sorghum (Daneshvar et al., 2003; Oller et al., 2006; Wang et al., 2013). These components include highly toxic and persistent substances and due to exceptional reactivity threaten to jeopardize the aquatic environment through agricultural circle and washing (Fenoll et al., 2012a; MacBean, 2012).

Many different methods for removing pesticides from water are known, and we especially investigated photocatalytic degradation. TiO_2 as a photocatalytic semiconductor is the most suitable chemical compound for removal of harmful substances from the environment

by photocatalytic process. Its chemical inertness, stability to the photo and chemical corrosion, as well as low price is its advantages as a catalyst (Lambropoulou et al., 2011). Photocatalytic degradation is based on the irradiation of UV light which results in the generation of oxidative species that are characterized by high and non-selective reactivity, so they can easily attack and decompose the molecules of organic pollutants. Photon has energy which is greater or equal to the band gap energy of semiconductor (TiO_2). In that way electron (e–) from the valence band (VB) excites to the conduction band (CB), leaving a positive hole (h+) behind. The energy level at the bottom of the valence zone effectively reduces the potential of photoelectrons, while the peak energy of the valence zone creates its ability to oxidize. Electrons and cavities migrate to the surface of the catalyst and reduce species present on its surface. Photogenerated cavities may oxidize organic molecules or react with OH^- ion, or H_2O, oxidizing them to $^{\bullet}OH$ radicals. Photogenerated electrons can also react with oxygen, translating it into superoxide anion $O_2^{-\bullet}$ radical. This reaction leads to additional formation of $^{\bullet}OH$ radicals. These radicals as very strong oxidative agents having the ability to oxidize organic pollutants adsorbed on the surface of TiO_2 as mineral products (Verma et al., 2013).

Nicosulfuron and dimethenamid-P have been photodegradated using photocatalytic degradation (Fenoll et al., 2012a; Fenoll et al., 2013; Halle et al., 2010; Sarmah & Sabadie, 2002). TiO_2, as a most effective photocatalyst was used (Malato et al., 2002; Fenoll et al., 2012b), and the effect of different operational parameters was investigated (Malato et al., 2002; Ahmed et al., 2011; Burrows et al., 2002). The purpose of present work is to study the photocatalytic degradation of insecticides nicosulfuron and dimethenamid-P in water using TiO_2 as catalyst. The effect of parameters such as initial concentration of catalyst, initial pesticides concentration, initial salt concentration (NaCl and Na_2SO_4), and pH were studied.

MATERIALS AND METHODS

All chemicals used in the investigation were of reagent grade and were used without further purification. Nicosulfuron (technical grade, 98.1%, Galenika-Fitofarmacija, Serbia) was applied without further purification. Dimethenamid-P (purity min. 99%) was supplied by Riedel de-Haën (Seelze-Hannover, Germany). Titanium dioxide (TiO_2) labeled as P25 supplied by Evonik was used in experimental part of the work. The photodegradation of nicosulfuron and dimethenamid-P were studied by preparing solutions containing 20 mg/L of nicosulfuron and 34.5 mg/L of dimethenamid-P.

RESULTS AND DISCUSSION

Photocatalytic degradation of nicosulfuron

The photocatalytic degradation of nicosulfuron in deionized water was performed and the influence of reaction parameters to degradation of pesticide were investigated. The duration of exposure of nicosulfuron solution to irradiation was set to 90 minutes. In our experiments we used the most common semiconductor photocatalyst TiO_2. The experiments were performed with 1g/L, 2g/L, 3g/L, 4g/L, and 5g/L of TiO_2. The disappearance rate in the process of photocatalytic degradation can be described by a pseudo-first kinetic order, as shown by the Eqs. (1-2):

$$\ln\left(\frac{C_0}{C}\right) = k \cdot t \qquad (1)$$

$$C = C_0 \cdot e^{-k \cdot t} \qquad (2)$$

where k – is the reaction rate constant [mg/L·min]; C_0 – is the initial pesticide concentration and C – is the pesticide concentration in time t.

For nicosulfuron degradation the optimal concentration of the catalyst was found to be 4 g/L (Figure 1). The effect of the initial nicosulfuron concentration on the degradation rate (r) is given in Eqs. (3-4).

$$r = \frac{K \cdot k_C \cdot C}{1 + K \cdot C_0} = k \cdot C \qquad (3)$$

$$\frac{1}{k} = \frac{1}{K \cdot k_C} + \frac{C_0}{k_C} \qquad (4)$$

where K and k_C are the Langmuir–Hinshelwood (L-H) adsorption equilibrium constant [L/mg] and the rate constant of surface reaction [mg/L·min], respectively. According to Langmuir-Hinshelwood model, the values K and k_C were found to be 0.0555 L/mg and 1.492 mg/L·min, respectively. At the investigated concentrations, i.e. the concentrations up to 20 mg/L the applicability of the L–H equation for photocatalytic degradation was confirmed by the linear plot (R^2=0.979) (Figure 2).

Both salts (NaCl and Na_2SO_4) inhibit photocatalytic degradation (Figure 3).The results imply that the photodegradation rate was highest at PZC at pH 6-7 (Figure 4). The ion chromatography (IC) results showed that mineralization led to the formation of nitrate, nitrite and ammonium ions during the process. Mineralization of organic carbon was incomplete and about 69% of initial TOC disappeared after 90 minutes. These results are in accordance with those of other researchers (Fenoll et al., 2012a; Fenoll et al., 2013).

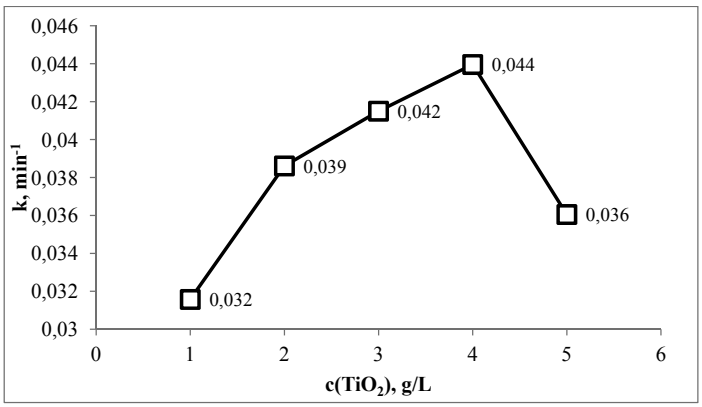

Figure 1. Influence of the initial concentration of catalyst (1-5 g/L) on the photodegradation rate of nicosulfuron (c = 20 mg/L) irradiated with Osram Ultra vitalux lamp (315-400 nm).

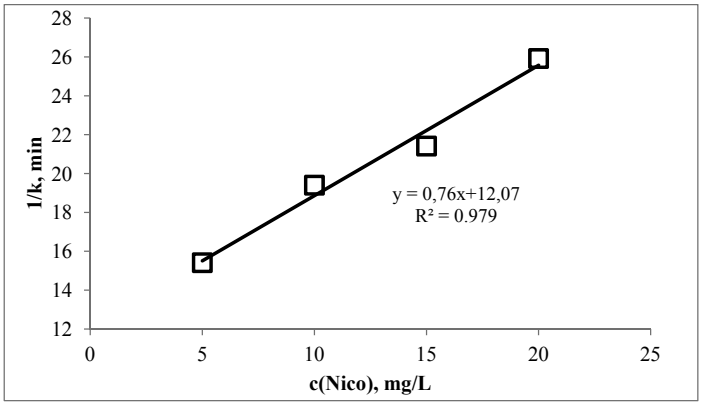

Figure 2. The effect of initial pesticide concentration (5-20 mg/L) on the photodegradation rate of nicosulfuron with TiO_2 (c = 4 g/L).

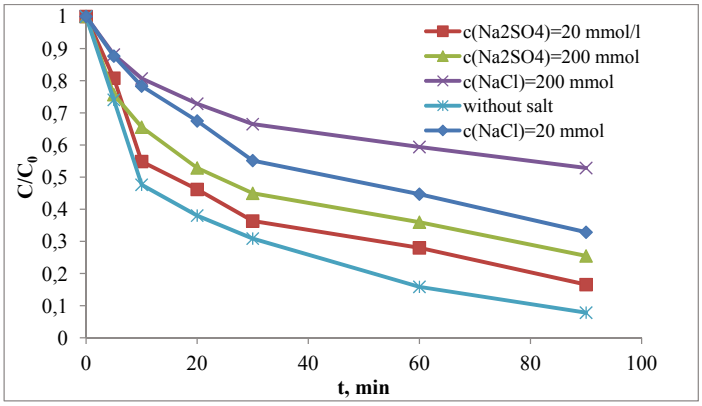

Figure 3. Salt effect (20-200 mM) on the photodegradation rate of nicosulfuron (c = 20 mg/L) with TiO_2 (c = 4 g/L).

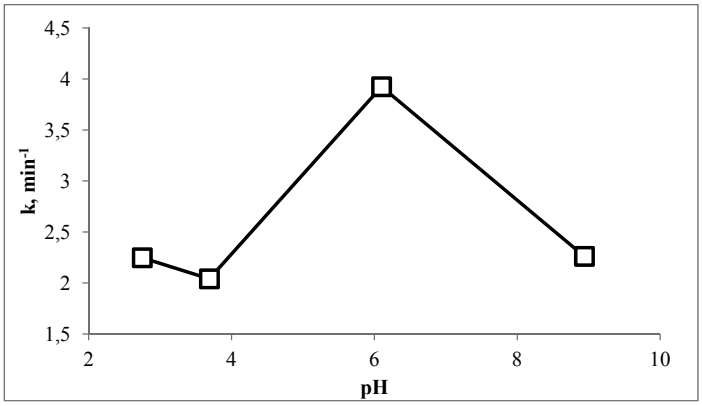

Figure 4. The effect of pH (1-12) on the photocatalytic degradation rate of nicosulfuron (c = 20 mg/L) with TiO_2 (c = 4 g/L).

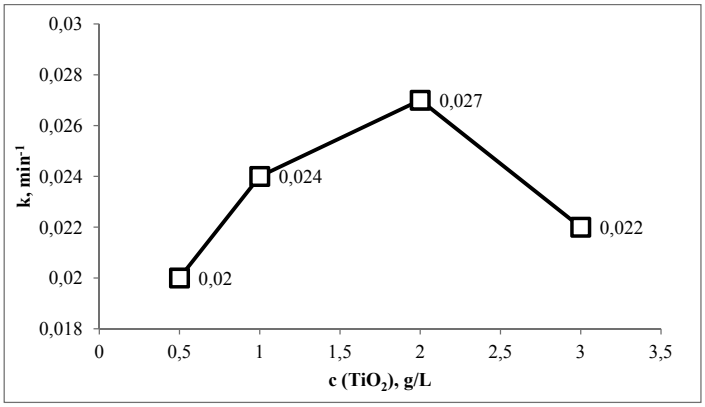

Figure 5. Influence of the initial concentration of catalyst (0.5-3 g/L) on the photodegradation rate of dimethenamid-P (c = 34.5 mgL^{-1}) irradiated with Osram Ultra vitalux lamp (315-400 nm).

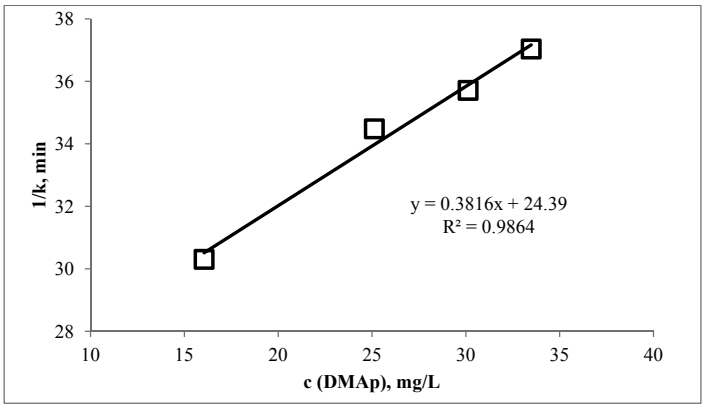

Figure 6. The effect of initial pesticide concentration (5-50 mg/L) on the photodegradation rate of dimethenamid-P with TiO_2 (c = 2 g/L).

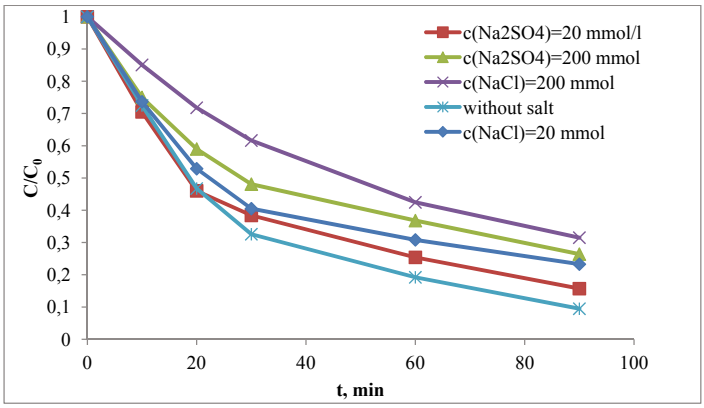

Figure 7. Salt effect (20-200 mM) on the photodegradation rate of dimethenamid- P (c = 34.5 mg/L) with TiO_2 (c = 2 g/L).

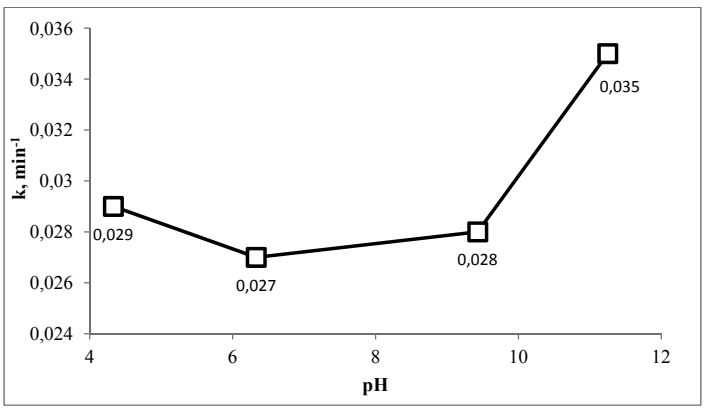

Figure 8. The effect of pH (1-12) on the photocatalytic degradation rate of dimethenamid-P (c = 34.5 mg/L) with TiO_2 (c = 2 g/L).

Photocatalytic degradation of dimethenamid-P

The experiments were performed with 0.5g/L, 1g/L, 2g/L, and 3g/L of TiO_2 and for dimethenamid-P the optimal catalyst concentration was 2g/L (Figure 5). Also, we investigated the influence of initial dimethenamid-P concentration, and the reaction followed Langmuir-Hinshelwood mechanism Eqs(1-4), pseudo-first order (Figure 6). The values for K and k_C were found to be 63.92 L/mg and 0.041 mg/L·min. Both salts act like inhibitors (Figure 7). The increase of initial insecticide concentration conducted to decrease of dimethenamid-P degradation rate and the photodegradation rate of dimethenamid-P was higher in alkaline then in acidic conditions (Glavaški et al., 2014) (Figure 8). After 90 minutes of irradiation 98% of dimethenamid-P was degraded. About 63% of initial TOC disappeared after 90 minutes and mineralization led to the formation of nitrate, sulfate and ammonium ions.

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DEVELOPMENT OF HERBICIDE FORMULATIONS BASED ON QUIZALOFOP-P-ETHYL

Slavica Gašić, Ljiljana Radivojević, Dragica Brkić, Marija Stevanović and Anđelka Tomašević

Institute of Pesticides and Environmental Protection, Banatska 31b, Belgrade-Zemun, Serbia
slavica.gasic@pesting.org.rs

ABSTRACT

Pesticides are formulated in many different ways. Quizalofop-P-ethyl is selective post emergence herbicide which can be found on the market formulated as emulsifiable concentrate (EC) and suspension concentrate (SC). The objective of this investigation was to develop two types of formulations: emulsifiable concentrate (EC) and went a step forward and developed oil in water (EW) formulation. Oil in water (EW) is a kind of pesticide formulation which contains water instead of almost all organic solvents. EW recently replaces EC as a new excellent environmentally-friendly pesticide formulation. We started investigation with development of EC formulation (50 g a.s./L) and after that developed EW formulation with the same concentration of active ingredient. The results obtained by testing developed formulations according to FAO and WHO recommendations shown that they had necessary stability to be applied in plant protection.

Key words: herbicide, emulsifiable concentrate (EC), emulsion oil in water (EW)

INTRODUCTION

The active ingredient in an herbicide formulation is the chemical that controls the target weed. The herbicide product is usually made up of active ingredients mixed with inert to allow dilution, application, and stability. The mixture of active and inert ingredients (solvents, emulsifiers, adjuvant etc.) is called a formulation. A single active ingredient often is sold in several different kinds of formulation. The formulation can have a major impact on the effectiveness of a product, including how well it mixes and performs in various environmental conditions and what influence has on users and environment. The primary purpose of formulation is to optimize the biological activity of the pesticide, and to give a product which is safe and convenient for use (Knowles, 2005).

Quizalofop-p-Ethyl, [Ethyl (2R)-2-[4-(6-chloroquinoxalin-2yloxy) phenoxy] propionate] is selective post emergence herbicide which control annual perennial grass weeds and can be used in different crops. This active ingredient can be found on the market formulated as emulsifiable concentrate (EC) and suspension concentrate (SC) (MacBean, 2012). The presence of organic solvents in EC formulations can lead to safety hazards in use and to a negative impact on the environment generally. Recently the solvents which are used in EC formulations have come under toxicological and subsequently regulatory pressure. As a results some of the most common solvents are no longer available. On the other side oil in water (EW) is a kind of pesticide formulation with appearance of milky white liquid which is formed by dispersing liquid pesticide or disolved solid pesticide mixed with inert in water. Since the formulation contains water instead of almost all organic solvents, EW recently replaces EC as a new excellent environmentally-friendly pesticide formulation (Mulqueen, 2003; Knowles, 2008).

The objective of this study was to investigate the possibility of developing formulations such as EC and EW starting from Quizalofop- P-Ethyl as active ingredient in concentration of 50 g/L.

MATERIALS AND METHODS

Quizalofop-P-Ethhyl technical material (95% min.) was originate from Sinochem Ningbo, P.R China. All reagents and solvents were purchased from commercial sources and used without further purification. Emulsifiers which were used were of commercial quality (Ajinomoto OmniChem, Belgium and Rhodia, Milano, Italy) and were used without further purification.

The content of active ingredient Quizalofop-P-Ethyl was determined by high performance liquid chromatography (*HPLC*) using ultraviolet detection (UV) (Helwett Packard HP 1050 liquid chromatorgraph).

Particle size distribution was measured by CILAS 1064 liquid and visual aspect of formulations checked by Axioskop 40 (Carl Zeiss, 63x Canon camera).

The pH was controlled by CIPAC method MT 75; Density CIPAC method MT 3; Persistent foaming CIPAC MT 47.2; Storage stability CIPAC MT 39.3 and MT 46.3 (Dobrat, Martin, 1995).

EW formulations are unstable systems, therefore special attention was focused to stability tests and that measurement were prolonged to ensure that developed EW formulation is stable enough to be used in plant protection.

Emulsifiable concentrate (EC) of Quizalofop-P-Ethyl was prepared by mixing solvent (Solvesso 100), emulsifiers (6%) and active material (5%). For homogenization magnetic stirrer was used.

Emulsion oil in water (EW) of Quizalofop-P-Ethyl was obtained by progressively adding oil phase in water phase under stirring. Oil phase was prepared with active material (5%), esterified rape seed oil (10%), Solvesso 100 (40%) and mixture of emulsifiers (8%), while water phase was prepared with water, antifoam agent (0.2%) and monopropylenglicol (5%) as antifreeze. Oil phase was adding into water phase under high shear mixing. For homogenization Ultra turrax mixer (speed 8000 o/min, duration 15 minutes) was used.

RESULTS AND DISCUSION

The objective of this investigation was development stable formulations of emulsifiable concentrate and oil in water emulsion with Quizalofop-p-Ethyl as active ingredient. Emulsifiable concentrate represent oil solution of active ingredient altogether with emulsifiers. This type of formulation is generally applicable for active ingredients which are soluble in organic solvents such as herbicide Quizalofop-p-Ethyl. This is an old type of formulation and its development is relatively simple. EC formulations are designed to be added to water and to be applied after emulsification (Mollet, Grubenmann, 2001). Emulsifers are adding to the formulations to ensure good emulsification after dilution with water. In investigation we started for initial screening of suitable solvent for the technical material and proper emulsifiers based on knowledge of active ingredient and emulsifiers. After selection of solvent and emulsifiers the right balance between them was found by experimentation which aim was development of stable emulsion to provide the desired results later during application. The samples were stored at controlled temperatures in order to evaluate their stability over the time. Among different samples which were prepared it was found the best solution on the basis of stability and physico-chemical properties. The results of chosen formulation are given in the Table 1. The obtained results indicated that the formulation remained stable after stability test; content of active ingredient was changed from 46.3 g/L (5.16%) to 46.7 g/l (5.20%), density 0.8963-0.8977 g/cm^3, pH 4.8-5.0 (stable region for this active ingredient), persistent foaming 20-22 cm^3 (which is not going be a problem for application) and stability after 0.5h and 2h were good and reemulsification was complete.

Table 1. Physical and chemical properties of fresh EC formulation and after stability tests

Time	fresh formulation		after 7 days		after 14 days	
Temperature	room temperature		0^{0}C		54^{0}C	
Content of Quizalopf-P-Ethyl	46.3 g/l		46.4 g/l		46.7 g/l	
Density	0.8973 g/cm^3		0.8963 g/cm^3		0.8977 g/cm^3	
pH (1% in distilled water)	5.0		4.8		5.0	
Persistent foaming	22 cm^3		20 cm^3		20 cm^3	
Stability of emulsion and reemulsification	0.5h	0/0	0.5h	0/0	0.5h	0/0
	1h	0/0	1h	0/0	1h	0/0
	2h	0/0	2h	0/0	2h	0/0
	24h	1/0	24h	2/0	24h	2/0
	REE	0/0	REE	0/0	REE	0/0

The difficulty in preparing oil in water formulation is that the formulation belongs to thermodynamically unstable system. Actually oil in water formulation composed of two phases: dispersed oil phase with active ingredient and a continuous water phase. The active ingredients for this type of formulation must be liquid (or dissolved solids in organic solvent) and oil phase must have a low water solubility. The proper choice of oil and emulsifiers is very important to avoid different kind of stability problems such as flocculation and coalescence which are the major routes of emulsion degradation. Suitable solvent should be immiscible or at least with low solubility, in water and oil solution should be stable to crystallization at different temperatures during storage. We prepared the formulation by dissolving the active ingredients (Quizalofop-P-Ethyl) in oil phase (solvesso 100) and the oil solution is than emulsified into water phase. Suitable emulsifiers for this type of formulations are those which form and stabilize oil-water emulsions. Emulsifier by forming electric double layer on the surface of the dispersed phase and steric hindrance effect of interfacial film keep stability of emulsion (Tadros, 2005). For stabilization we used nonionic blended emulsifiers as they have good performance and have wide range of application. In this study, the optimum emulsifiers content were determined through a series of tests. EW requires high-strength shearing, stirring and homogenization to prepare technical material (active ingredient) into 0.5-1.5 μm droplets, and make it disperse in aqueous solution and maintain the state of stable storage (Gašić et al., 2006; 2012). For homogenization we used Ultra turrax mixer which is strong enough to serve a purpose. The particle size distribution was controlled by observation with optical microscope (Figure 1) and by light scattering as it is critical for the stability of emulsion. A narrow particle size distribution is necessary to achieve as it will ensure better stability than a wide particle size distribution (Zhang, 2014). In particular, the storage stability of formulation should meet the guidelines of the Manual on development and use of FAO and WHO specification for pesticides (Anonymous, 2010). In accordance with this manual the content of active ingredient in the formulation is not allowed to decline by more than 10% when formulation stored at room temperature over a period of two years. The shelf life of oil in water formulation can be predicted by the measurement of the active ingredient content and a series of physical parameters before and after storage tests. Storage at temperature of 0^0C (seven days) and 54^0C (14 days) is used to control physical and chemical stability. To test the storage stability samples of the formulation under development are stored for a specific time in tightly sealed glass vessels at the different temperature indicated in each case (Tables 3 and 4). Special attention was focused on particle size distribution as the most important parameter for this type of formulation (Table 5). The samples are subsequently examined and compared with the value of fresh prepared formulation at the beginning of the storage (Table 2). As the method designed to predict shelf life might be inadequate to ensure a robust product in storage we prolonged stability test to make sure that we will have stable formulations.

Table 2. Physical and chemical properties of fresh EW formulation

Aspect	Milky liquid	
Content of active material Quizalopf-P-Ethyl:	51.0 g/l	
Density	0.9486 g/cm^3	
pH (1% in distilled water)	5.5	
Persistent foaming	5 cm^3	
Particle size Distribution (Mean diameter)	1.04 μm	
Stability of emulsion and reemulsification	0.5h	0/0
	1h	0/0
	2h	0.5/0
	24h	2/0
	REE	0/0

Table 3. Physical and chemical properties of EW formulation after stability test at 0°C

Test period	7 days		30 days
Content of active material Quizalopf-P-Ethyl:	49.5		48.3 g/l
Density	0.9537 g/cm^3		0.9507 g/cm^3
pH (1% in distilled water)	5.5		5.8
Persistent foaming	1 cm^3		0 cm^3
Particle size Distribution (Mean diameter)	0.99 μm		0.98 μm
Stability of emulsion and reemulsification	0.5h	0/0	1/0
	1h	0/0	1/0
	2h	1/0	1/0
	24h	2/0	1.5/0
	REE	0/0	1/0

Table 4. Physical and chemical properties of EW formulation after stability test at 54°C

Test period	14 days	90 days
Content of active material Quizalopf-P-Ethyl:	49.5	48.4 g/l
Density	0.9535 g/cm^3	0.9535 g/cm^3
pH (1% in distilled water)	5.4	4.9
Persistent foaming	5 cm^3	6 cm^3
Particle size Distribution (Mean diameter)	0.80 μm	0.90 μm
Stability of emulsion and reemulsification	0.5h 0/0	0/0
	1h 0/0	0/0
	2h 0/0	1/0
	24h 1/0	2/0.1
	REE 0/0	0/0.1

The results showed that particle size distribution varied from 0.80 μm to 1.04 μm which means that even after prolonged stability tests particle size distribution remind fine which indicated that emulsion will be stable. Variation of density was 0.9486 g/cm^3 to 0.9537 g/cm^3; pH value varied from 4.9 to 5.8 and at these pH values the active ingredient is stable, as it stable in neutral and acidic media (MacBean, 2012) Persistent foam varied from 0 cm^3 to 6.0 cm^3. Visual aspect of aqueous diluted EW formulation showed very fine droplets (Figure 1). The content of active ingredient varied from 48.3 g/l (5.08% to) to 51.0 g/l (5.34%) and this differences are considered to be acceptable (Anonymous, 2010). Stability of emulsion was good after 30 minutes and one hour and reemulsification complete.

It can be concluded that two different types of formulations were prepared using Quizalofop-P-Ethyl as the active material. First developed emulsifiable concentrate (EC) is common way of commercially formulation of this active ingredient, but oil in water (EW) emulsion represent new formulation solution for this herbicide. Emulsion oil in water as water base system has various advantages over EC formulation (e.g. safety aspect) which meet recent demands of regulatory authorities and the pesticide industry.

Fig.1. Aspect of aqueous dilute oil in water (EW) formulation of Quizalofop-P-Ethyl

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Table 5. Particle size distribution after prolonged stability tests for EW formulation

	Diametar at 10% (μm)	Diametar at 50% (μm)	Diametar at 90% (μm)	Mean Diametar (μm)
fresh formulation	0.72	0.97	1.47	1.04
stability test - 7 days at 0±2 ^{0}C	0.67	0.93	1.42	0.99
stability test - 30 days at 0±2 ^{0}C	0.64	0.92	1.42	0.98
stability test - 14 days at 54±2 ^{0}C	0.45	0.76	1.21	0.80
stability test - 90 days at 54±2 ^{0}C	0.53	0.85	1.34	0.90

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