Phomopsis (Diaporthe) & Spinach Seed: History, Concerns, & Research Update

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• Since 2011: 11 spinach seed lots restricted at U.S. ports of entry
• Organic & fungicide-treated
• USDA APHIS inspectors:
  • large, black structures visible with 'naked eye'
• Microscopic examination: *Phomopsis*
• DNA sequencing by APHIS mycologists:
  • *Diaporthe viticola*
  • *Diaporthe melonis var. brevistylospora*
  • *D. eres* (Holland)
• 1st two = exotic to USA, therefore quarantine status
Diaporthe viticola

- NOT the teleomorph of *Phomopsis viticola*
- Weak grapevine pathogen: Germany & Portugal
- Select references:
**Diaporthe melonis var. brevistylospora**

- **Melon & grape pathogen in Japan**
  - Ohsawa & Kobayashi (1989):
    - postharvest concave rot of melon fruit in Japan
    - beta conidia <20 um long
    - anamorph = *Phomopsis brevistylospora*
  - Kinugawa et al. (2008): Berry drop of grape in Japan

- **D. melonis** – cucurbit pathogen in U.S.
  - beta conidia >20 um long
  - anamorph = *Phomopsis cucurbitae* (Beraha & O'Brien, 1979)

**Diaporthe eres**

- Butternut (*Juglans*), maple, & peach in U.S. & E.U.
- NOT quarantine fungus in U.S., found on seed in E.U.
Impacts

• Estimated value of rejected lots: >US$20M?
  - + Seed lots voluntarily inspected in Holland, not shipped?
  - Effects on U.S. spinach seed supply?
  - 11 lots intercepted, only 4 had species verified by APHIS

• Conference calls, 2012-13

• Proposal prepared in Oct. 2012 (ASTA request)
  • Submitted to APHIS PPQ on 2 Nov. 2012
  • APHIS PPQ response received on 28 Jan. 2013
  • Comments/conference calls, proposal revised Feb. 2013
    • Methods, priorities, where research will take place, permits for handling seed & isolates

• Taxonomy of *Phomopsis* & *Diaporthe* spp.
  • APHIS mycologists: ITS, TEF, TUB2, & RPB2 sequences
ASTA Spinach *Phomopsis* Proposal

1. Test spinach seed lots for *Phomopsis*
   - E.U. & U.S. lots, sampling/assay protocols
     - visual, microscopic, & DNA sequencing
     - 0.01-0.03% incidence
     - 1 lot for each quarantine species for Objective 2

2. Seed treatments
   - Conventional & organic, registered & potential
   - Create ~50% incidence
   - Seed health (freeze-blotter), germination (blotter), & growout assays

3. Test isolates for pathogenicity
   - spinach, grape, soybean, melon, ...

**Collaborators:** WSU, Univ. of Arkansas, Sakata Seed America, ASTA seed companies, Germain's, USDA APHIS
Screening of spinach seed lots

- Received samples of 6 spinach seed lots with 'suspect' black fungal structures
- All 6 lots were grown in Denmark
- No lots produced in USA identified with *Phomopsis* prior to 2014 season
- Manually examined individual seeds of each lot with magnifying lens
- Suspect seed: slide mounts, seed incubated, isolations on agar media from cirrhi on pycnidia
- Wait for pycnidia to form & mature on media
- Isolates with *Phomopsis* pycnidia/conidia sent to APHIS PPQ mycologists
- Need ≥5,000 infested seeds/lot to generate 50% incidence/lot for seed treatment evaluations
**Diaporthe viticola**

**Diaporthe melonis var. brevistylospora**
Seed treatments

- Conventional & organic
- ~50% infested Lots A & F
- **Seed health assay**: 4 reps of 100 seeds/treatment
- **Germination assay**: 4 reps of 100 seeds/treatment
- **Grow-out assay**: 4 reps of 100 seeds/treatment
- Lot F first, then test most effective treatments on Lot A
## Lot F (*D. melonis var. brevistylospora*) treatments

<table>
<thead>
<tr>
<th>#</th>
<th>Seed treatment</th>
<th>Active ingredient</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Hot water</td>
<td>50°C water</td>
<td>20 min, cold water rinse 3x, dry</td>
</tr>
<tr>
<td>3</td>
<td>Chlorine</td>
<td>1.2% NaOCl</td>
<td>40 min, rinse 3x, dry</td>
</tr>
<tr>
<td>4</td>
<td>KleenGrow</td>
<td>7.5% didecyl dimethyl NH₄Cl</td>
<td>30 s in 11.7 ml/liter water, dry</td>
</tr>
<tr>
<td>5</td>
<td>Tsunami 100</td>
<td>15.2% peroxyacetic acid + 11.2% hydrogen peroxide</td>
<td>30 min in 9.5 ml/liter water, dry</td>
</tr>
<tr>
<td>6</td>
<td>Oxidate 2.0</td>
<td>27.1% hydrogen dioxide + 2.0% peroxyacetic acid</td>
<td>2 min in 10 ml/liter water, dry</td>
</tr>
<tr>
<td>7</td>
<td>Seed Support II</td>
<td>Proprietary</td>
<td>Proprietary</td>
</tr>
<tr>
<td>8</td>
<td>Apron + Thiram 42-S</td>
<td>Mefenoxam + thiram</td>
<td>187 + 7.5 g/100 kg seed</td>
</tr>
<tr>
<td>9</td>
<td>Farmore D300</td>
<td>Mefenoxam + fludioxonil + azoxystrobin</td>
<td>68 + 48 + 2.5 g/100 kg seed</td>
</tr>
<tr>
<td>10</td>
<td>Farmore D400</td>
<td>Mefenoxam + fludioxonil + azoxystrobin + thiabendazole</td>
<td>68 + 48 + 2.5 + 50 g/100 kg seed</td>
</tr>
<tr>
<td>11</td>
<td>Apron + Thiram + Rancona</td>
<td>Mefenoxam + thiram + ipconazole</td>
<td>7.5 + 187 + 10 g/100 kg seed</td>
</tr>
<tr>
<td>12</td>
<td>Apron + Coronet</td>
<td>Mefenoxam + boscalid + pyraclostrobin</td>
<td>0.32 fl oz + 0.38 fl oz + 0.76 fl oz/100 kg seed</td>
</tr>
</tbody>
</table>
Spinach seed Lot F: Seed germination assay
(D. melonis var. brevistylospora)
Spinach seed Lot F: Seed health assay (D. melonis var. brevistylospora)

• Protocol:
  - Freeze-blotter assay: assess fungicide efficacy
  - Control seed = non-treated
  - % of seed with black pycnidia = viable + non-viable
  - % of seed on which pycnidia produce cream-colored cirrhi = viable pycnidia

• 2 reps (100 seed/treatment): Freeze-blotter assay
  - 48.0% of seed with Phomopsis pycnidia
  - 2.5% of seed with ‘viable’ pycnidia (cirrhi) - other, rapid-growing fungi prevented observation of cirrhi
  - 84.0% Alternaria, 69.0% Verticillium, 35.0% Fusarium, 30.5% Stemphylium, 20.5% Colletotrichum

• 4 reps: NP-10 agar assay (60 s NaOCl for control seed)
Spinach seed Lot F: Seed health assay (21 d) (D. melonis var. brevistylospora)

% of seed infected/infested

- Control
- Hot water
- Chlorine
- Kleengrow
- Tsunami 100
- Oxidate 2.0
- Seed Support II
- Apron+Thiram
- Farmore 300
- Farmore 300+Merflect
- Rancon+Apron+T...
- Apron+Coronet

Verticillium
Alternaria
Colletotrichum
Fusarium
Stemphylium
Spinach seed Lot F: Seed health assay (21 d) 
(D. melonis var. brevistylospora)
Seed treatment summary

• Lot F (*D. melonis* var. *brevistylospora*) germ assay:
  - Non-treated seed = 95% germination
  - 50% *Phomopsis* & other fungi did not reduce germination even after storing the seed >2 years
  - Seed Support II reduced germination to 69% (+ 20% abnormal germination & 10% non-germinated seed)
  - 1.2% NaOCl for 40 min increased germination by 4%

• Lot F health assay:
  - Pycnidia only observed on 37% of control seed by 21 d because many other fungi overgrew *Phomopsis*; only 10% with viable pycnidia by 21 d
  - Seed Support II & chlorine eradicated pycnidia initially, & kept the % seed with pycnidia at 21 d to less than the control seed
Seed treatment summary

- **Lot F health assay (ctd):**
  - 4 of 5 organic disinfectants increased % seed with viable *Phomopsis* pycnidia observed - killed competing fungi?
  - Seed Support II reduced % seed with viable *Phomopsis* pycnidia to ~1%, but also reduced seed germination
  - 6-17% of seed treated with hot water or conventional fungicides had viable pycnidia
  - No treatment completely prevented development of *Phomopsis*, but NP-10 assay reduces fungicide efficacy
  - Infection appears fairly deep-seated

- **Lot A (*D. viticola)*: limited seed, waited to hear from APHIS PPQ about 2013 CA paper of this species on olive trees; APHIS PPQ: seed treatment must be 100% effective to be accepted for imported seed
Pathogenicity test of isolates of the 2 quarantine species

- Jim Correll, University of Arkansas
- APHIS PPQ permit
- 9 isolates of *D. viticola* (Lot A)
- 16 of *D. melonis* var. *brevistylospora* (Lots D & F)
- Tested on agar media: pycnidia/conidia formation

**Not pathogenic on:**
- spinach
- grape cuttings

**To be tested on:**
- melon
- soybean
Summary

- **Seed treatment evaluations with Lot F:**
  - 50% *Phomopsis* & other fungi did not affect germination adversely even after storing seed >2 years (95% germ)
  - Seed Support II reduced germination to 69%
  - 1.2% NaOCl for 40 mins increased germination by 4%
  - Seed Support II reduced viable *Phomopsis* to 1% of seed
  - No treatment eradicated *Phomopsis*, but NP-10 agar assay limited fungicide efficacy

- **Pathogenicity testing:**
  - *Spinach & grapevine cuttings*: None of 29 isolates caused symptoms, regardless of inoculation method
  - *Melon, soybean, ...*: to be tested

- **Diaporthe eres `species complex` on 2014 WA seed lots**: 7 isolates (+3) = unknown species (“lack of molecular data”)
Summary

• Urbez-Torres et al. (2013):
  - Plant Disease 97:231-244. Olive twig and branch dieback: Etiology, incidence and distribution in California.
  - *D. viticola* & other species cause dieback of olive trees

• Brendon Reardon, APHIS PPQ, 18 Dec. 2014:
  "...rejected import shipments ... were deemed to be infected with an exotic fungal disease that can cause germination loss and seed decay... the APS paper ... does make note of *D. viticola*; however, at this time, APHIS and the U.S. Customs and Border Protection will continue to regulate pests and pathogens that are concluded to be infesting and infecting spinach seed, respectively, including *D. viticola, D. melonis* var. *brevistylospora*, and "*D. viticola-like" and “*D. melonis-like" organisms, until and unless they can be proven to not be regulated. I know you appreciate that the taxonomy of this species complex is not well understood.”
Questions?