



# Cornell Institute for Biology Teachers

Copyright CIBT, 1999.

This work may be copied by the original recipient from CIBT to provide copies for users working under the direction of the original recipient. All other redistribution of this work without the written permission of the copyright holder is prohibited.

Lab issue/rev. date: 4/30/02

**Title:**

## The Science of Rotting Grocery Produce

**Authors:**

David Kalb, Cornell University, Dept. of Plant Pathology, Ithaca, NY 14853, and Bill Bandura, Niskayuna High School, Niskayuna, NY 12309.

**Appropriate Level:**

This lab is appropriate for Life Science, High School, Honors, or AP students.

**Abstract:**

Students will investigate the bacteria (plant pathogens) that cause soft rot on grocery produce (that mushy mess you often see on vegetables). By bringing some of this rotting produce from the store back to the lab students can isolate bacteria that are responsible for the disease (and sometimes yeasts, other fungi and other bacterial saprophytes). The following laboratory write-up is divided into two main groups:

- 1. Isolation**-where students isolate the organisms present in the rotting vegetable using a variety of techniques. Using two different methods of dilution plating (or streaking), colonies of the pathogen should be visible a few days later growing on nutrient media. The concepts of population levels, dilution, microenvironment, saprophyte, pathogen, ecological niches and others can all enter into the discussion.
- 2. Koch's Postulate**-where students grow larger quantities of some of the bacteria they isolated and test them for the ability to cause the same symptoms as those observed on the host. A few or many isolates can be tested. Suspect colonies are chosen and transferred to separate Petri dishes and streaked out to grow. Suspect colonies are inoculated on healthy slices of cucumber, whole or slices of potato, etc., to observe if what they isolated is capable of causing soft rot. The concepts of proper environment (mainly humidity and temperature) need to be addressed.

**Time Required:**

Actual laboratory work can be accomplished in under one hour on the first day. The second part can be done in an additional hour (approx.) period 24-72 hours later. More time needed after for observation and recording results and follow-up discussions.

**Living Environment:**

**1- Analysis, Inquiry, and Design:** 1.1- Purpose of inquiry: 1.1a; **4- Living Environment:** 5 – Dynamic Equilibrium: 5.2a,b 6- Interdependence; 6.1g

## Additional Teacher Information

- When obtaining veggies from the store, peppers are one of the best to do the isolations from. The soft rot symptoms on peppers are easy to spot. Cucumbers are also good followed by carrots and potatoes. The symptoms on the fruit are sunken, soft, areas that may or may not be associated with breaks or tears in the skin. The areas will look “water soaked” and changed in color. The produce department of any local supermarket would be glad to give you the rotten stuff. Call ahead. Much of what they want to give you may not be saleable but it will not have soft rot. When you get some “good” rotting veggies, you can refrigerate them for a day or two if needed.
- Once a good pathogenic isolate is obtained, it can be stored in water in the freezer or refrigerator for years. Make a sterile water blank in a sterile plastic or glass vial and add a few loop-fulls from a 24-48hr pure culture. You can then inoculate your own fruit or veggies and save a trip to the store.
- When testing for pathogenicity, use noninoculated controls. Explain what a control is and why it is used in experiments.
- Information with which students should be familiar: general lab safety, using microburner, alcohol, etc., sterile techniques (Why do you flame the transfer loop? Why do you keep the lid on the Petri dish?), pectinase and cellulase enzymes (how do bacteria feed?), the economic importance of soft rot.

**Materials:** A suitable growth medium dispensed into Petri dishes such as nutrient agar or potato dextrose agar, at least 4 per student (more depending on whether Koch’s Postulate is tested), sterile water, sterile empty Petri dishes, sterile transfer pipettes, scalpels or razor blades, transfer loops, rotting peppers from the store and healthy potatoes (and/or peppers, cucumbers to check for a successful isolation-Koch’s Postulate), paper towels, plastic bags and plastic incubation containers.

### Supplemental Reading

Agrios, G. N. *Plant Pathology*, 4<sup>th</sup> ed. Academic Press San Diego CA, 1997.

# **The Science of Rotting Grocery Produce**

## **New York State Learning Standards**

### **Standard 1: Analysis, Inquiry and Design:**

Key Idea 1: The central purpose of scientific inquiry is to develop explanations of natural phenomena in a continuing, creative process

1.1 - laborate on basic scietific andpersonal explanations of natural phenomena, and develop extened visual models and methematical formulations to represent one's thinking.

a. Scientific explanations are built by combining evidence that can be observed with what people already know about the world.

### **Standard 4: The Living Environment**

Key Idea 5: Organisms maintain a dynamic equilibrium that sustain life.

5.2 - Explain disease as a failure of homeostasis.

a. Homeostasis in an organism is constantly threatened. Failure to respond effectively can result in disease or death.

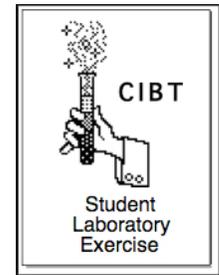
b. Viruses, bacteria, fungi, and other parasites may infect plants and animals and interfere with normal life functions.

Key Idea 6: Plants and animals depend on each other and their physical environment.

6.1 - Explain factors that limit growth of individuals and populations

g. Relationships between organisms may be negative, neutral, or positive. Some organisms may interact with one another in several ways. They may be in a producer/consumer, predator/prey, or parasite/host relationship; or one organism may cause disease in, scavenge, or decompose another.

# The Science of Rotting Grocery Produce



## Isolation of Plant Pathogens

While in most cases a plant disease can be diagnosed by observing the signs of the pathogen or the symptoms of the disease on the plant, occasionally it is necessary to isolate the pathogen from the diseased tissue and grow it in pure culture. The organism, thus isolated, can then be identified, and, if necessary, the cultures can be used to inoculate healthy plants to confirm the pathogenicity of the organism.

Isolation involves freeing the pathogen from plant tissue, which is usually relatively easy (with the exception of obligate parasites) and also freeing it from contamination by other microorganisms, which is often very difficult. Contaminants are everywhere - adhering to the surfaces of the plant or even colonizing (as **saprophytes**) the tissues killed by the pathogen you are trying to isolate. Bacterial cells and fungal spores can be found clinging to the surfaces of your skin, clothing, and hair. They can even be carried aloft on air currents and settle with the dust on virtually all the surfaces in the lab, from which they can be lofted again to contaminate your glassware, instruments and culture vessels. Therefore, to minimize the chance of these beasts "finding their way" into your cultures, we have to practice techniques that go well beyond mere cleanliness.

**Aseptic (or Sterile) Technique.** There is no one "right" way to exercise good aseptic technique; different people have their own preferred approaches. The object is to reduce the odds of contamination to within acceptable limits. Obviously acceptable limits of contamination for us are much higher than in a surgical lab, for example, and we do not have to take the extreme precautions of scrubbing and gowning. There are, however, a few simple practices that we can adopt:

1. Sterilize all culture media in an **autoclave** and use glassware and culture vessels that either have been autoclaved or are sterile, disposable containers packaged in a sterile protective wrap.
2. Avoid drafts that will carry air-borne spores into your open culture vessels or stir them up from nearby surfaces to later settle into your work.
3. Before starting to work, clean the tabletop and other surfaces that may have collected dust and spores. Spread a large, moistened cloth over the work surface to trap spores that come in contact with it and prevent them from lofting again.
4. Tie back your hair, roll up your sleeves, and wash your hands before you begin.
5. Open sterile containers (tubes, flasks, Petri plates, *etc.*) carefully, and if possible, keep the openings partially covered. Keep them open only as long as necessary.
6. Sterilize transfer needles and loops by heating them to redness. Cool them by touching the sterile agar or sterile water into which you will be transferring the culture.
7. Sterilize tools that cannot be heated to redness (scalpels, forceps, scissors, *etc.*) by immersing them

for **at least 5 minutes** in 70% ethanol. The ethanol should be flamed off before the instrument is used. (Note: The **alcohol** does the sterilization in this case; the flaming merely burns off the alcohol. The temperatures achieved are too low to effectively kill all microorganisms on the surface of the instrument.)

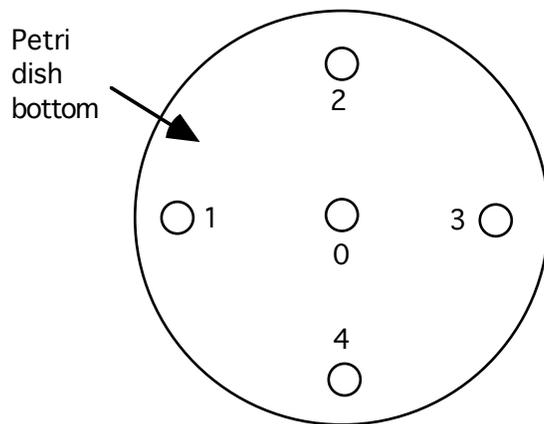
8. If flasks or tubes have cotton or plastic foam stoppers instead of caps, flame the mouth of the vessel immediately after removing the stopper to kill any adhering spores that could slip in as contaminants.
9. Do not set down on a contaminated surface anything that must remain sterile. Hold plugs and caps in your hand while making transfers.
10. Seal Petri plates with Parafilm strips to minimize the risk of aerial contamination around the edge of the plate.
11. Do not store fresh plant material or soil in the same place you are trying to maintain pure cultures. Mites (with all sorts of contaminants clinging to their bodies) can easily crawl through cotton stoppers and other kinds of closures.

***Isolating Bacteria—General.*** Where bacteria are present in large numbers in tissues, they often can be easily isolated by macerating the tissues in a few drops of sterile water. It is sometimes a good idea to first kill the contaminants on the surface of the tissue by immersing it in a dilute hypochlorite solution and rinsing it in sterile water, but this step is not always necessary and more often used for isolation of fungal pathogens. The central tissues of a lesion are usually in a more advanced stage of decay and may be invaded by a number of secondary organisms. The chances of getting the primary pathogen in pure culture are much better right at the margin of the lesion. Excise a very small piece of tissue such that about half of it is apparently healthy tissue and half is diseased.

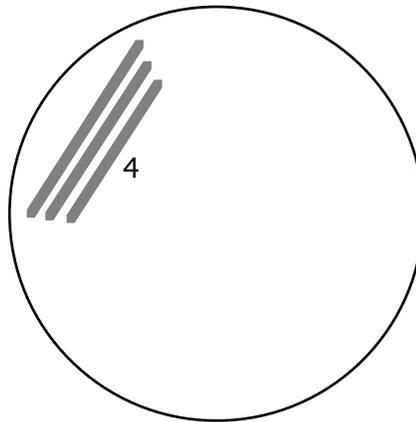
Because the pathogenic soft rot bacterium is usually present in high numbers, it can be isolated from the contaminating organisms by dilution. Do the isolation following one or both of the methods outlined below. After 24-48 hours examine the plates, pick out discrete colonies that look like the predominating organism, and streak them individually on nutrient agar. If Koch's Postulate is going to be tested, always use fresh bacteria to do the inoculation (24-72 hours old). Plates of bacteria can be stored in the refrigerator for weeks but a new plate should be started after storage. The organism most often responsible for causing soft rot is *Erwinia carotovora*.

### **Isolating Bacterial With Method #1**

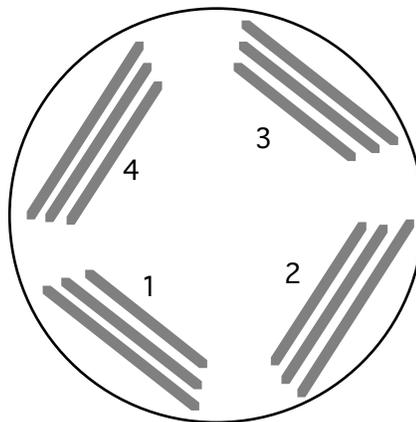
1. Clean your work area, wash your hands and prepare your tools as described above. Label two Petri dishes (simply called "plates" in the jargon of plant pathologists) containing nutrient agar (NA) with your name, the date, and "soft rot, potato" or something similar.
2. Prepare an empty, sterile, plastic Petri dish by drawing 5 small circles on the underside (outside) as shown in the figure below, one in the center and the others evenly spaced around the perimeter. Label the center circle "0" and the others, in sequence, "1" through "4".



3. With a sterile pipette, place a single drop of sterile water (approx. 0.1ml) above each of the circles inside the Petri dish.
4. Select one of the veggies with bacterial soft rot. Note the odor, color and consistency of the rotted tissues.
5. Remove a layer of surface tissue if appropriate (which may be contaminated with many different microorganisms), and with a sterile scalpel cut a small piece of tissue (about 2 mm on a side) from the **margin** of the rotted area thus exposed.
6. Place the rotted tissue in the drop of water in the center of the Petri dish and chop it finely or crush it with the scalpel. Let it stand for 2-3 minutes while you prepare a transfer loop.
7. Flame a transfer loop to redness and hold it in the air for a minute to allow it to cool. Put the loop in the drop containing the macerated tissue and stir it around for a few seconds.
8. Pick up a loopfull of the suspension and place it in the drop numbered "1" and stir it around for a few seconds. Pick it up and place it in drop 2, stirring it as before. Pick it up and place it in 3, and so on until you have 4 drops, each containing a bacterial suspension roughly 1/10 the concentration of the one before it.
9. Flame the loop to redness again to kill any possible undiluted bacteria that might be clinging to it. Allow it to cool in the air for about a minute.
10. Now sampling the drops **in reverse order beginning with number 4**, pick up a loopfull of water and carefully and lightly streak 3 parallel lines along the margin of the plate in the pattern shown in the figure below.



11. Without flaming the loop again, pick up a loopfull from drop 3 and streak it in 3 parallel lines at right angles to the first. Repeat the procedure for drops 2 and 1, streaking each set of 3 at right angles to the previous set, leaving you with the pattern shown below.



12. Seal the plates with strips of Parafilm, as demonstrated by the instructor, and place them inverted in your drawer to incubate. Record your observations during the next 24-48hr.

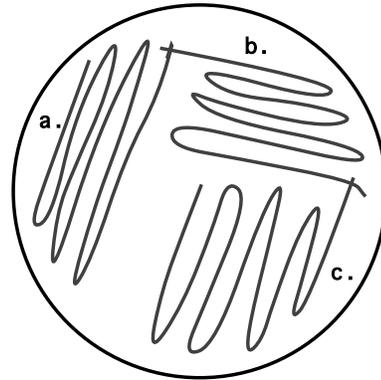
### **Isolating Bacteria With Method #2**

1. Wipe the work area with a disinfectant (10% Clorox).
2. Place 2-3 drops of sterile water in one spot in a sterile Petri dish.
3. With a scalpel, cut out 1 small (about 5mm<sup>2</sup>) piece of diseased tissue from the margin of the lesion (next to the necrotic tissue) and transfer it to the central drop of sterile water in the sterile plastic Petri plate.
4. Thoroughly mince and crush the tissue in the water using a sterile (flamed) scalpel. Allow the crushed

tissue to stand in the water for 2-3 minutes to allow separation of the bacteria from plant tissue.

6. Using a flamed, cooled, transfer loop, pick up a loop-full of suspension and streak a plate of agar according to the scheme below:

- a. Streak the loop of suspension lightly over the surface of about 1/3 of the plate to make many lines close together. Do not let the lines overlap.
- b. Flame and cool the loop again and draw it across the end of the previously streaked area 2 or 3 times and streak about 1/4 more of the plate.
- c. Again flame and cool the loop and draw it across the area you just streaked 2 or 3 times and streak the remaining 1/4 of the plate. Do not let the lines overlap.



This procedure progressively dilutes the bacteria as they are streaked across the plate. You should get solid lines of bacteria from the first streak, but individual colonies by the end of the third streak. Since the pathogenic bacterium should be present in large numbers and contaminants should be present in comparatively low numbers, only the pathogen may be present in the most dilute part of the streak. The contaminants should be diluted out.

7. Label your streaked plate and incubate it upside down at room temperature. Observe the plate each day until separate individual colonies approximately 2 mm in diameter are present. Did you get good separation of colonies? Do all the colonies look alike?

## Proof of Pathogenicity

Known diseases can be diagnosed by recognition of the characteristic symptoms and signs and by isolation and identification of the pathogenic organism(s), if any are involved. Diagnosis of a "new" or unknown disease requires a more rigorous procedure. If a living agent is isolated from the diseased plant, its pathogenicity (that is, its ability to cause the observed disease) must be established. The logical steps of such a proof were set down by J. Henle in 1840, but were first applied experimentally by R. Koch in 1883 and so are known as **Koch's Postulates**. In brief, they are:

1. By observation, the organism must be constantly associated with the disease in all affected individuals.
2. The organism must be isolated from the diseased individual and grown in pure culture.
3. When inoculated into a healthy individual, the organism must cause the disease originally observed.
4. The same organism must be reisolated from the inoculated individual.

Many secondary invaders and saprophytes are excluded by the first postulate, but often they are isolated along with the pathogen. Proper isolation technique and the right culture media are necessary. Inability

to isolate an organism might mean only that techniques were faulty. Thus it may be necessary to try several techniques. The same is true for the third postulate; the wrong method of inoculation or nonconducive environmental conditions could cause a negative result, despite the pathogenicity of the organism. The use of healthy 'control' inoculations is also important, especially in procedures for postulates 3 and 4.

Koch was a bacteriologist working on a disease of mammals. In general, his procedure is as straightforward and effective for bacterial and fungal diseases of plants as it was for that disease. With an array of other biotic agents that cause plant diseases, however, problems may arise. Viruses, viroids, and phytoplasmas may be difficult to find in diseased plants or may be present, without causing overt symptoms, in what appear to be healthy plants. Many plant pathogens cannot be grown in pure culture or require special culture media. Some pathogens infect only certain tissues or depend on specific insect vectors, so adequate and consistent inoculation may be difficult to obtain. Although the postulates tend to suggest that there is only one pathogen for each disease, in some cases a disease may result from the interaction of the plant with two or more pathogens. If such a possibility is not recognized, successful diagnosis of the disease may be hindered by strict adherence to the postulates.

Nevertheless, the proof of pathogenicity, which was important historically in the development of plant pathology, is useful and is used. Any issue of the journal Plant Disease includes descriptions of "new" diseases for which the role of a pathogenic organism has been established according to Koch's postulates. (See also Agrios, pp. 34-35.)

The purpose of this exercise is to demonstrate the relevance of Koch's postulates by testing the pathogenicity of the organism isolated from the infected vegetables.

## **Procedure**

1. Soak potatoes overnight in water to increase their moisture content.
2. Prepare a disinfested work surface as you normally do. Select a potato and disinfest the surface using 0.5% sodium hypochlorite (10% chlorine bleach) or 70% ethanol for this.
3. With a sterile toothpick (99% of the time, an unsterilized toothpick will work just fine), make two puncture wounds into the tuber about 2cm deep at least a few inches apart. Push the toothpick up and down a few times to enlarge the hole and macerate tuber tissue. Label one puncture "A" and the other "B".
4. With the toothpick from "A", pick up as much bacteria from the isolate to be tested as possible and push it into the preformed hole. Leave it in place. The toothpick from "B" becomes the noninoculated control. Leave it in place also. Take a very wet paper towel and wrap the potato up, place it in a plastic bag, seal it and place it in a warm environment (27-30°C) out of the sun. Soft rot symptoms can be seen in as little as 48 hours but best after 4-7 days depending on temperature and humidity. Symptoms are often seen with rot visible at the surface. It may or may not be accompanied by foaming and frothing. It is sometimes necessary to cut the potato in half to see if soft rot has occurred.

## **An Alternate Method**

1. Make 1 cm slices from a surface-disinfested potato tuber or cucumber. Place them in a tight sealing

plastic container into which very moist paper towels have been laid. Take a loopfull of the bacteria to be tested and smear it over the surface of the vegetable slices. Incubate as above. A daily spray of sterile water may be needed to prevent things from drying out. Make sure to do a few noninoculated slices also. Maceration of tissue with this method is generally quicker and can be easily visualized. Experiments that vary the temperature or humidity can easily be performed.