INQUIRY & INVESTIGATION

Investigating Plant Pathogen Responses: Using a Common Moss and a Soil Pathogen to Demonstrate Plant Defense Mechanisms

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Abstract

Plant–pathogen interactions are often omitted as a topic in most introductory and upper-level biology courses. The infection process of the plant pathogen Pythium irregulare *on the moss* Mnium cuspidatum *can be observed and exploited to provide lessons on host–pathogen responses, as well as introduce other biological topics such as microscopy, spectrophotometry, and enzymes. Students can qualitatively analyze plant responses to pathogen infection using microscopy and observe quantitative enzyme responses to draw conclusions. Students are also encouraged to generate hypotheses and test them using this biological system as a method to develop scientific skills.*

Key Words: *microscopy; assay; spectrophotometry; moss; plant pathogen; hypersensitivity response.*

○ Introduction

Although host and pathogen interactions often are discussed in the lecture content of many introductory biology courses, very few laboratory exercises provide students with opportunities to engage with and inquire about them. We present a novel classroom activity for students to study these interactions in a safe and interesting way by observing the hypersensitive response in plants via simple microscopy techniques and a colorimetric assay. Using mosses as a model organism for observing pathogen response makes exposing the plant to the pathogen and observation of the outcomes convenient, because mosses are easy to grow in limited space and can be viewed under a microscope without manipulation (most of the plant body is only a single cell layer thick).

Plants, like other living things, can fall prey to pathogenic organisms in their environment.

Plants, like other living things, can fall prey to pathogenic organisms in their environment. Similarly to animals, plants respond to pathogen attack by using a number of defense strategies. One strategy includes innate immunity, which is characterized by preestablished physical and chemical barriers, such as thick cork, strong cell walls, and a waxy cuticle. Secondarily, induced immunity is characterized by changes in the physical barriers, the appearance of new chemical deterrents, and alterations in gene expression that elicit additional defense responses (Oliver et al., 2009).

One of the major induced responses is the hypersensitive response (HR). HR is a complex set of immediate physiological changes that the plant undergoes in the area imminent to infection (Kombrink & Schmelzer, 2001). Upon pathogen challenge, the physical barriers around an infection site are altered through reinforcement of the cell wall, which potentially thwarts the movement of the pathogen through the plant (Ponce de León et al., 2012). In addition, production of chemical deterrents such as phenolic compounds, hydrogen peroxide, and other reactive oxygen species is induced (Quan et al., 2008). These deterrents damage cellular aspects of the pathogen such as membrane lipids, proteins, and genetic material, which may slow or kill the pathogen. Many of these aspects of HR are mediated by a class of enzymes called peroxidases (Almagro et al., 2009). Peroxidases use the substrate hydrogen peroxide to donate electrons to other cellular components that can activate them for a response. A generalized equation for a peroxidase catalyzed reaction can be simplified to the following:

$$H_2O_2 + B \rightarrow O_2 + BH_2$$

Electrons pass from hydrogen peroxide to compound B that reduces and activates it. For example, peroxidases can cross-link cell wall components together, are involved in phenolic compound synthesis, and can generate reactive oxygen species such as oxygen radicals, additional hydrogen peroxide, and hydroxyl radicals (Almagro et al., 2009).

HR is induced because plants are able to sense the presence of a pathogen using cell-surface receptors for pathogen-specific molecules.

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These molecules are referred to as defense elicitors and are often released by the pathogen (Garcia-Brugger et al., 2006). One example of an elicitor is the polysaccharide chitosan, a fungal cell wall component. When a plant is exposed to chitosan, it will secrete its endogenous peroxidase stores to initiate HR within the cell walls of the plant (Ponce de León & Montesano, 2013). This response can easily be detected using various stains and light microscopy to directly observe the affected tissues.

○ Objectives

This experiment has three main objectives:

1. Observe typical physiological responses of plants to fungal-like pathogens using microscopy to make qualitative observations.
2. Collect quantitative data on peroxidase activity in plant tissue using an enzyme assay.
3. Analyze data using Microsoft Excel and basic statistics.

This lab exercise is appropriate for high school classes or college courses such as general botany or cell biology; it can be adjusted for difficulty level and availability of materials to illustrate various biological phenomena tailored to the course objectives. Prelab assignments could require students to research background information on related topics, including the evolutionary history of land plants, enzyme kinetics, signal transduction pathways, host–pathogen relationships, gene-for-gene resistance, innate immunity in plants, HR in plants, and plant pathogens.

○ Materials List

Moss: *Mnium cuspidatum*

Mnium cuspidatum is endemic to North America and can be found easily in understory areas around trees or near buildings, growing in large clumps with other mosses (Figure 1). Sterile cultures can be obtained by collecting and sterilizing outdoor mosses with standard procedures (Smith, 2002). Axenic cultures of *M. cuspidatum* can also be obtained by contacting researchers actively working on this species. The moss is grown on BCD medium (Ashton & Cove, 1977). Other mosses locally available can be substituted.

Figure 1. *Mnium cuspitadum*.

Pathogen: *Pythium irregulare*

Pythium irregulare can be obtained from plant pest diagnostic labs often associated with state agriculture extension offices. It is a common soil pathogen and can be sent within state lines without hindrance. The pathogen is grown on PDA medium (Sigma) and Difco Bacto agar (Carolina). *P. irregulare* is safe and easy to contain since it is an oomycete that does not produce airborne spores; however, anything exposed to the pathogen, such as forceps and containers, should be autoclaved to prevent the spread of the pathogen to other plants.

Microscopy Materials

- compound light microscope
- fluorescence microscope (optional)
- 0.5% toluidine blue
- lactophenol blue (200 g phenol, 0.5 g cotton blue, 400 mL glycerol, 200 mL lactic acid, 200 mL deionized water)

Enzyme Assay Materials

- spectrophotometer (or microplate reader, optional)
- chitosan (Sigma)
- guaiacol (Sigma)
- hydrogen peroxide, 3%
- aqueous phosphate buffer (25 mM $NaPO_4$, pH 6.5)
- deionized water
- test tubes
- 1.5 mL microcentrifuge tubes
- test tube shaker (optional)
- pipettes and pipette tips
- mortar and pestles or tissue grinders

○ Procedure

Culturing Moss and Pathogen

M. cuspidatum is grown in sterile culture using BCD medium under fluorescent lights (16/8-hr light–dark cycle). *P. irregulare* is grown in a petri plate with 1/8 strength PDA medium supplemented with additional agar; to make 500 mL of 1/8 strength PDA, add 2.4 g of PDA and 6.5 g of Difco Bacto agar. Reduced-strength medium is needed to maintain the ability of the pathogen to infect the plant (personal observation). For all infection experiments, use actively growing pathogen collected from the growing front of a PDA plate.

Experiment 1: Microscopy (~30 min)

Simple and quick microscopy techniques can be used to visualize the HR in plants by inoculating plant tissue with the pathogen 24 h prior to observation. During the inoculation period the moss

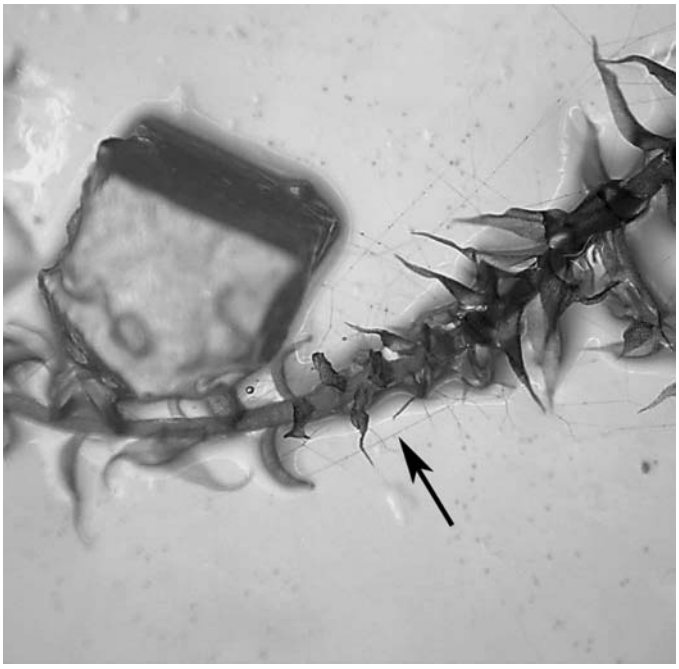

Figure 2. Inoculation technique. Note the PDA plug used to inoculate the moss with the pathogen. Thin strands of pathogen hyphae can be seen coming off of the moss.

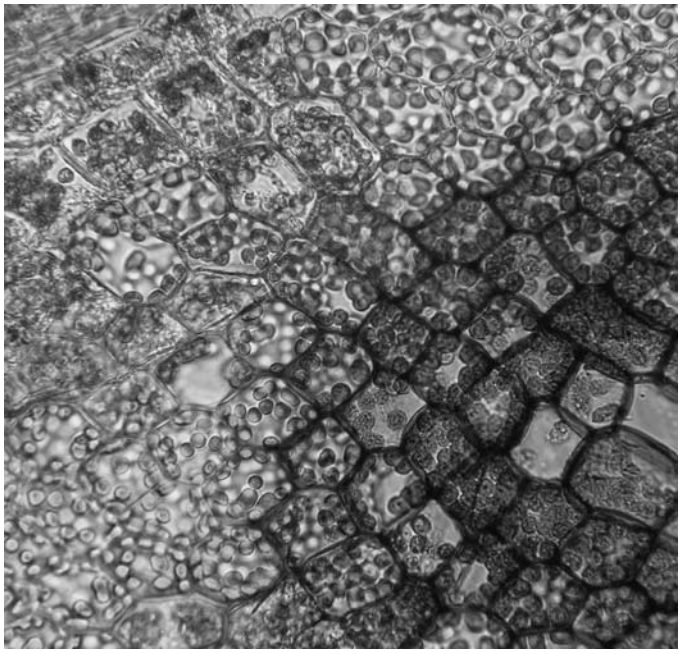

Figure 3. Toluidine blue staining of hypersensitive response (HR) in *M. cuspidatum*. Blue indicates changes in cell wall polysaccharides; purple indicates presence of phenolics.

with pathogen is kept under the moss growing conditions mentioned above. Using a scalpel, take a small agar piece (1 mm^3) containing the actively growing *P. irregulare* culture and place it on the distal end of a moss thallus (Figure 2). Let the culture stand for 24 h. To view the pathogen, immerse an infected sample of the moss in lactophenol blue for several minutes. Mount on a microscope slide with water and a coverslip, then observe under a compound microscope. The stain is taken up differentially by the pathogen and not the plant. To view HR, remove an additional sample of infected tissue and immerse it in 0.5% toluidine blue stain for 5 min, and then destain with water. Mount with water and coverslip, then observe under a compound microscope. Note the appearance of dispersed chloroplasts within uninfected cells and aggregated chloroplasts within infected cells (Figure 3). Toluidine blue is a polychromatic dye and can be used to visualize two hallmarks of HR (Ponce de León et al., 2012). First, changes in cell wall polysaccharides, an indication of wall strengthening, are observed as areas staining blue in color (Figure 3). Second, the induction of phenolics (antimicrobial agents) is observed as areas staining purple in color (Figure 3).

Additionally, if a fluorescence microscope is available, unstained infected tissue can be observed for the HR. Autofluorescence of chloroplasts occurs normally in uninfected tissue, whereas chloroplast autofluorescence is absent in infected tissue. Furthermore, autofluorescence of phenolic compounds can be observed within infected regions, but it is absent in uninfected regions.

Experiment 2: Colorimetric Assay of HR Enzyme Activity (~60 min)

In this exercise, peroxidase enzymes associated with HR are screened collectively for their activity with an assay using a consistent concentration of a known substrate, guaiacol. As the reaction occurs, peroxidases transfer electrons from hydrogen peroxide to guaiacol, turning from clear to orange-brown as it is reduced. Because increasing enzyme concentration increases the rate of the reaction, a simple colorimetric assay for differences in light absorption between substrate and product can be used to quantify the release of moss peroxidases that mediate HR induced by the pathogen elicitor.

Prior to the experiment, prepare solutions of 2 mg/mL chitosan and 25 mM phosphate buffer pH 6.5 in deionized water. pH 6.5 is necessary to maintain the correct environment necessary for enzyme function in this experiment. On the day of the experiment, prepare a substrate solution of 10 mL of deionized water, 30 µL of H_2O_2 (3%), and 10 µL of guaiacol.

To conduct the experiment, each student group should collect approximately 40 mg of moss material. Material collected from culture should be free of agar and patted dry. Completely submerge plant material in a total volume of solution (in mL) equal to 20 times the sample weight (in mg) in test tubes. Two treatment groups should be made: submerge half of the sample in deionized water (control) and the other half in a working solution of 1 mg/mL chitosan (experimental); this can be accomplished by first loading half of the total volume with water and then the other half with a stock solution of 2 mg/mL chitosan. (Chitosan is not very soluble in water and should be mixed well before and during use.) Leave the samples in their treatments for 5 min on a test tube shaker, if available (Figure 4). Remove the samples from the test tubes and quickly rinse them in fresh deionized water. Quickly grind the samples in phosphate buffer, with a volume (in mL) equal to 40 times the sample weight (in mg) to produce endogenous enzyme samples. Transfer samples into 1.5-mL microcentrifuge

tubes and centrifuge at 16,000 g for 2 min. Transfer 160 µL of the supernatant into a test tube and dilute the sample with 1840 µL of deionized water.

To determine the relative concentration of peroxidase enzyme within the moss, mix the endogenous enzyme samples with the guaiacol substrate in the ratio of 1:2 volumes. To do so, load 500 µL (enzyme) and 1 mL (substrate) of this reaction mixture into a cuvette, and immediately begin measuring the absorption of the solution with a spectrophotometer set to 490 nm every 30 to 60 sec for 6 min. Students can work in groups so that at least three control and three experimental treatments are analyzed.

Figure 4. *M. cuspidatum* completely submerged in a treatment solution.

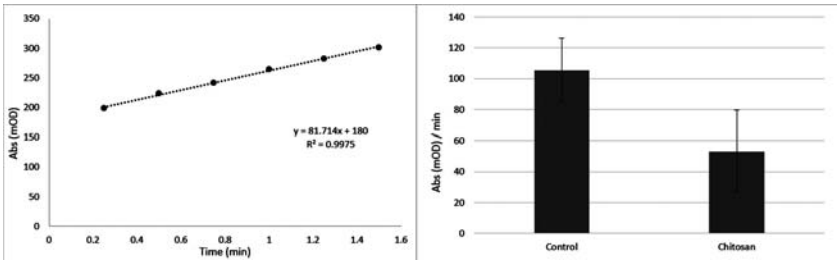

Figure 5. Example data set. (Left) Determining peroxidase reaction rate by plotting absorption of product over a timecourse. (Right) Example of compiled rates between control and treatment groups ($N = 5$).

Enter the data points into an Excel worksheet and generate a scatterplot of the points (Figure 5). Plot absorption on the y-axis and time on the x-axis. Display a linear trendline for the data. Select to display the equation for the trendline and R^2 value. The R^2 value indicates how linear the curve is. The slope of the line should be recorded as a data point for statistical analysis. The difference in control and treatment in Figure 5 is a reflection of the cell releasing peroxidases in response to the presence of a fungal elicitor. Basic statistical analysis, such as a Student's *t*-test, should be performed to determine if the control rates are significantly different from the rates acquired from the treatment samples (Figure 5).

Procedure Variations

This lab protocol can be modified in different ways to provide students with opportunities for open-ended inquiry. After the students have learned the simple lab procedures described above, the following procedure options can be presented to students to give them the opportunity to ask questions, formulate hypotheses, generate experiments, collect data, and analyze results. Procedure modifications may include a comparison with another elicitor or plant hormone, a time-course study, whole-plant staining procedures for the release of oxygen radicals, or the use of a microplate reader for the enzyme assay to increase sample size, as described below.

Visualizing the effects of different polysaccharide elicitors on moss: β-glucan (among others) is another well studied plant pathogen elicitor that can be compared against chitosan with respect to the timing and strength of HR. Furthermore, plant pathogen response hormones, such as salicylic acid, or even hydrogen peroxide itself, could be substituted for the elicitors. Students could ask whether different elicitors result in similar responses by the plant, or whether known plant defense hormones control peroxidase release.

Timing of HR: Timepoints for peroxidase activity can be taken anywhere from 1 to 30 min after pathogen infection. Students could ask questions regarding speed, duration, or peak timing of the response following infection.

Localization of oxygen radicals during HR: Hydrogen peroxide can be localized in whole plant tissues by incubating the plant with 3,3′-diaminobenzidine (DAB). Oxygen radicals can be revealed within tissues by staining whole plants with nitroblue tetrazolium (NBT). Students could ask questions regarding whether oxygen radicals are produced solely at the infection site or more diffusely across the plant.

Microplate variation: If a microplate reader is available, students can examine how microquantities and automation can influence enzyme kinetics data collection and analysis. The following general procedure can be used: Prepare the samples as outlined above. Then, transfer 80 µL of the supernatant into a fresh cuvette and dilute the sample with 920 µL of deionized water. Load 50 µL of enzyme and 100 µL of substrate into each microplate well and immediately begin measuring the absorption of the solution with a spectrophotometer set to 490 nm every 15 to 30 sec for 6 min.

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