A ny experienced biology teacher knows that when it comes to catching students’ attention there is nothing like the topic of infectious disease. Studying viruses and other pathogenic organisms is a favorite activity in most introductory classes for the obvious reason that it is one to which everyone can immediately and instinctively relate. Moreover, courtesy of the popular press with its stories of flesh-eating bacteria and meningitis-carrying mosquitos, it is a subject that captures the imagination as well as the intellect. No other area in all of biology seems to generate as many (if frequently misinformed) questions, and when teaching its basic concepts and related ideas such as immunity, teachers have a unique opportunity in their classrooms to help students “experience the richness and excitement of knowing about and understanding the natural world” (National Research Council 1996).

However, with opportunity comes challenge, and one of many when presenting the fundamentals of pathology and immunology is that the very nature of the subject can make practicable investigations and experiments in the classroom problematic. Students can seldom observe the organisms and cellular mechanisms involved, even with a light microscope, and the liability issues alone make working with most pathogens in high schools nearly impossible. Consequently, there are few opportunities for students to study first hand the very subject teachers could use to excite them the most. Add in the fact that most current audio-visuals available on the topic look suspiciously like video games, and it is little wonder that the actual science behind disease remains a mystery to so many people. Teaching about it turns into an anthropocentric “Q & A” session about individual human illnesses, and students walk out of introductory biology courses without any real understanding of how central the issue of infection is to every living thing.

We would like to help change this situation. The tobacco mosaic virus (TMV) is a simple, safe, and readily available pathogen that teachers already use to model basic infectious disease in the classroom (McDaniel et al. 1998). We want to show how you can use it in conjunction with the common bean plant, *Phaseolus vulgaris*, to provide a discernable, experimental model with which students can explore an important topic in pathology and immunology: the concept of induced resistance. In particular, we want to show how to use TMV with *P. vulgaris* to demonstrate and study the practical consequences of “vaccination” — a topic normally requiring complex animal models not readily available (or legal) in a typical high school setting.
TMV & Systematic Acquired Resistance

TMV is a common pathogen of many modern crop species including tomatoes, tobacco, and the many varieties of the New World bean, P. vulgaris. The virus is known to produce systematic infections in its hosts by spreading throughout the organism from the initial site of entry (usually an injury) via the vascular tissues. In the case of P. vulgaris, it does its damage by causing numerous small lesions to form on the surface of an infected plant’s leaves (see Figure 1), thereby interfering with the organism’s photosynthetic mechanisms. While the virus seldom actually kills its host, it does stunt a plant’s growth and inhibit proper fruit development, and once infected, an organism must simply allow the disease to run its course; there is no real treatment (Kansas State 1997; Research School 1998; McDaniel et al. 1998).

Bean plants do not remain passive to a TMV attack, however, and like all multicellular organisms, P. vulgaris attempts to resist infectious disease with some form of immune response. When exposed to the pathogen, individual plants can quickly produce natural resistance compounds known as pathogenesis-related proteins and antibiotic-like chemicals called phytoalexins (Lyon 1999; Xiong 2000). Depending on the degree of exposure, the age and general health of a plant, etc., the infected organism will either produce a weak response and fail to prevent the disease from spreading, or it will produce a strong response and develop what is known as systematic acquired resistance (SAR).

SAR is still not a well understood phenomenon in plants, but researchers do know that it involves a significant increase in the production of pathogenesis-related proteins within almost all the cells of the exposed organism. It is also known that this increase creates a generalized barrier against the invading disease that essentially immunizes the plant against it (Zehnder et al. 1998). More importantly, researchers know that once SAR has been stimulated, it applies not only to the pathogen that induced it but to many others as well (Lyon & Newton 2000). Thus, unlike animal immune systems (which have to learn to recognize one disease at a time), plant immune responses are more generic, and a single SAR event can provide a plant with a lifetime of general immunity.

What is intriguing for our purposes, though, is that SAR can be triggered in the same fashion as the animal immune system by deliberate weak exposure to a pathogen. Inducing a small necrotic response to a virus such as TMV on the lower leaves of a bean plant can cause the remainder of the plant to become immune to further infection. Thus, just as it is possible to vaccinate animals against various diseases, we can “vaccinate” plants as well (Lyon et al. 1991). Even more interesting, we don’t even have to use the actual pathogen. A variety of elicitors can stimulate a strong response to a pathogenic infection in plants, resulting in SAR (Cohen et al. 1993; Reglinski et al. 1994; Lyon & Newton 2000). Common methods for doing so (in addition to prior inoculation of the lowest leaf) include treatments of commercial yeast extract, salicylic acid, and copper chloride (Reglinski et al. 1995; Laxalt et al. 1996; Vidal et al. 1997). Therefore, it is not only possible to elicit SAR against TMV in various host organisms such as P. vulgaris; it can be successfully done (and has been) without ever using the actual virus.

For the purpose of studying artificially induced immunity, then (as well as observing the natural course of a pathogen-host interaction), TMV and P. vulgaris provide an ideal model of infectious disease to examine. The range of questions you can have your students explore is almost endless: from simply looking at how inoculation works to comparing and contrasting animal and plant immune responses. Furthermore, the experimental protocol needed to study the model is completely accessible to everyone. TMV and P. vulgaris can make visible and touchable for our students a world otherwise hidden, and as we shall discuss later, the implications for learning about pathology and immunology are numerous.

The Protocol

Materials:

- 12 to 16 P. vulgaris plants
- 10 ml of 60% rehydrated lyophilized TMV
- 10 ml of 60% rehydrated tobacco sap
- 500 ml 0.0030 M salicylic acid solution
- Diatomaceous earth or Carborundum powder
- Eye droppers or transfer pipettes
- Distilled water
- 2 misting spray bottles
• Sterile cotton swabs
• Liquid dishwashing detergent
• Self-watering plant trays

Procedure

"Vaccination" Phase: Traditional

1. Divide your 12 to 16 adult plants into four treatment groups (A, B, C & D) so that there are 3 to 4 plants per group and label their containers accordingly; be sure to allow adequate space between individual plants since TMV spreads by direct contact.

2. For the plants in Group A, dampen a cotton swab with distilled water and dip into a container of diatomaceous earth. Being careful not to press down, gently wound the upper surface of the lowest leaf on all the plants by drawing the coated cotton swab back and forth across the surface of the leaf. Do not rub excessively or vigorously or hold onto the leaf while rubbing, or you will tear up the leaf surface beyond the plant's ability to repair itself and will get damaged leaves which will taint your data collection (see Figure 2).

3. Rinse off the diatomaceous earth with distilled water, and then use an eye dropper to apply 1 drop of the 60% rehydrated tobacco sap to the upper surface of each wounded leaf. These plants will serve as the negative control for the prior inoculation treatment.

4. For the plants in Group B, use the diatomaceous earth method described above to wound the lowest leaf of each plant. Then, after rinsing the leaves with distilled water, begin inoculation by using an eye dropper to apply 1 drop of the 60% rehydrated lyophilized TMV solution to the upper surface of each wounded leaf. These will serve as the experimental condition for the prior inoculation treatment.

"Vaccination" Phase: Nonpathogenic

5. For the plants in Group C, prepare a solution of 500 ml of distilled water + 1 drop of liquid dishwashing soap (to increase the even spread of the solution over the entire leaf surface). Then, using one of the spray bottles, mist the upper surface of all the leaves of all the plants, one squirt per leaf. These plants will serve as the negative control for the salicylic acid treatment.

6. For the plants in Group D, prepare a solution of 500 ml 0.0030 M salicylic acid solution + 1 drop of liquid dishwashing soap. Then, using the other spray bottle, mist the upper surface of all the leaves of all the plants, one squirt per leaf. These plants will serve as the experimental condition for the salicylic acid treatment.

Infection Phase

7. Following either "vaccination" treatment, wait 3 days to allow the experimental plants (Groups B and D) to acquire their systematic immunity. Then, using the diatomaceous earth method

Plants should be 12 to 14 days old following germination before beginning the experiment, and while we like to use the Great Northern variety of P. vulgaris, any of the subspecies will do. The virus and sap are available from Carolina Biological Supply Co., and the self-watering systems are available from either Ward or Boreal Labs as part of their Rapid Radish™ kits.

Almost all of these materials, though, can be purchased quite inexpensively at your local grocery and hardware store and made as follows. First, you can extract your own virus using 1 gram of cigar or cigarette tobacco and 10 ml of 0.1 M dibasic potassium phosphate. Simply grind the tobacco and the liquid in a mortar and pestle until the contents resemble the texture of cooked oatmeal (this may take up to 15 minutes of alternating soaking and grinding). Then dilute 1:100 and use the resulting liquid in place of the lyophilized virus. Use extra extraction fluid for your control. Next, you can build your own self-water system easily out of an old Styrofoam™ cooler. Simply cut the lid so that it just fits inside the walls of the cooler with about 5 cm of clearance on the ends; drape a piece of muslin or other heavy duty cotton cloth on the lid so that part of it hangs down in the cooler, and fill the cooler with water to just beneath the lid. Finally, you can substitute anti-wart medicine (like Compound W®) diluted to the proper concentration for the salicylic acid and 600-grit sandpaper for the diatomaceous earth/Carborundum powder.

A safety note: both diatomaceous earth and Carborundum powder are respiratory and skin irritants; so we recommend wearing goggles and protective gloves when using either of them. To decrease the risk of exposure further, we also recommend placing a small sample (no more than 1 to 2 grams) of the one you use in a covered petri dish and only opening the lid just enough to insert the dampened cotton swab to collect the sample you will use to wound the leaves.

Salicylic acid can be difficult to dissolve and preparing it can require vigorous agitation; be sure to add the soap after preparing the 0.0030 M solution.

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forms of statistical analysis on this information. Other equally meaningful and useful data can be amassed (McDaniel et al. 1998); we simply recommend you try different possible indices until you find the one that works best with your classroom situation.

Another significant issue as mentioned in Step 1 of the protocol is the physical environment of the plants described in Step 2, wound all the leaves on all the plants in Groups A through D except the originally wounded ones in Groups A and B. Again, remember not to wound too drastically or you will damage the leaves too much for the plants to recuperate.

8. After rinsing the leaves, use an eye dropper to apply 1 drop of the 60% rehydrated lyophilized TMV solution to the upper surface of each wounded leaf. Be sure to record the number of leaves infected per plant.

9. For the next two weeks, take daily observations which can include (but are not limited to): number of lesions per plant (see Figure 1 again), leaf size, plant height, and changes in coloration.

Discussion, Strategies & Suggestions

The variety and quantity of data students can collect from this experimental protocol are enormous, and one of the things we recommend from hard learned experience is to have your class focus on only one or two indicators rather than try to cover them all. As shown in Table 1, the number of lesions per plant can be a good measure of the severity of TMV infection, and the average ratio of lesions to “inoculation incidences” (how many leaves are exposed to the virus) can serve as a good gauge of the effectiveness of a particular “vaccination” method. Comparing the ratios of the control versus experimental groups, it is easy to see that the plants receiving the actual treatment suffer significantly less infection, demonstrating that both forms of preventative treatment can and will induce some level of SAR. These indices also make it easy for students to visualize their results readily in a graph (see Figure 3). Furthermore, the lesion-to-inoculation ratios provide an excellent opportunity to incorporate higher math skills into your lessons since it is possible to perform meaningful t-tests and other
plants so that as they grow and develop, the leaves of one plant do not come into physical contact with the leaves of another (especially between plants from different treatment groups!). Also, because small amounts of the virus erupt from the lesions it causes onto the leaf surfaces, it is equally important to prevent the accidental transfer of TMV via some direct instrument such as a hand or cotton swab.

To avoid both of these problems, we recommend placing the plants from each experimental condition on their own separate watering trays with a minimum distance of 5 cm between individual plants and a minimum distance of 20 cm between trays. This way, students can still easily access the individual plants for observational purposes with little risk of one plant brushing up against another, and at the same time, the amount of space needed for the experiment(s) to run is moderate. We also recommend that students thoroughly wash their hands and any tools they use (such as a ruler) between handling each plant and that they use any cotton swab only once before properly disposing of it. Doing so not only reduces the chance of an accidental physical cross-infection; it provides an excellent opportunity to teach the basics of sterile protocol. While one of the advantages of TMV is that it is an obligate plant pathogen (and hence poses no health hazard), it is still a good idea to get students into the habit of sound safety practices when working with either micro-organisms or pathogens.

Finally, we would like to offer some suggestions for possible ways to adapt and extend our protocol to examine a variety of medical issues beyond simply modeling “vaccination” and induced resistance. One obvious way, of course, is to have students explore what type of elicitors evoke the strongest immune response in bean plants. As evident in Figure 3, the salicylic acid treatment is significantly more effective than pre-exposure to TMV, and students could use the basic design of our protocol to examine the effectiveness of other known elicitors (copper solutions, yeast extracts, pretreated virus (exposed to various levels of heat to weaken or denature it), or even methods of their own invention (wounding, alternative chemical treatments, etc.). They could also modify the protocol itself to look at whether a different concentration of salicylic acid would work better or whether different methods of applying the “vaccination” treatments would be more effective (such as dipping the entire leaf into the test solution). In short, almost all of the issues that confront modern disease prevention (e.g. the weakened vs. dead polio vaccine debate; oral methods vs. injection; effectiveness vs. cost) can be examined using this one basic protocol, and only the imagination can limit its use.

Conclusion
A number of features of this activity make it attractive from the teaching standpoint. In addition to the ones discussed above, one of the most important is that the time outlay needed for it is relatively modest. The preparation of the materials takes about an hour, and while you will need about two days total class time to get the experiment running (a period to “vaccinate;” a period to infect), the rest of the experiment runs on its own. Students will need about 30 minutes each time they take their observations, but this process can be spread out over as much as a 3- to 4-week period if you wish, with minimal impact on the rest of your classroom schedule. Simply start things about a month before you plan to teach about pathology and immunology, and you will have all the fresh data you could ever need to engage your students deeply in learning how the immune response and the process of vaccination work.

We encourage you, though, not to limit your use of this activity to a “cookbook” approach. We think that there is tremendous learning potential here, and what we believe ultimately makes the described protocol so
appealing is its ability to pull so many different elements of biology together at the same time. For example, plant diseases that affect crop quality and yield are a major problem in our world today, often threatening famine and food shortages, and many of the common pathogens that cause them have developed resistance to current treatments. Furthermore, even where these treatments still work, the use of such “pesticides” is now usually frowned upon for environmental reasons, and so many scientists are currently studying various natural elicitors in the hope of creating benign, nontoxic crop protection systems that induce SAR and prevent the spread of disease without damaging the environment. Thus, just in this one activity, students encounter not only pathology and immunology but also evolution, botany, ecology, biochemistry and agriculture, and the list of significant biological concepts they could learn with it is potentially endless.

Therefore, the ultimate worth of our protocol, we believe, lies in its ability to provide students the chance to explore the whole multitude of biology’s many ideas and theories and to see how they intertwine to form our modern understanding of the natural world. Using it will enlarge your students’ understanding, and it will allow them to see the broader applications of their own knowledge – all while generating a lot of enthusiasm and excitement about science in general. Try it for yourself and see.

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