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Broad Spectrum Systemic Acquired Resistance in Amblystegium serpens

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in *Amblystegium serpens*

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of the Requirement for Graduation Honors

Christina A. Tatara
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Dedication

I would like to dedicate this thesis to Dr. Nat Hauck. You have been a fantastic mentor and teacher who through this research I can now fully appreciate the phrase “Serenity Now!”
Acknowledgments

I would like to thank Dr. Hauck for all his work and help in my research.

P. Winter, C. Bowman, and Dr. P. Villani for their previous work in defense mechanisms in A. serpens.

Dr. P. Villani for his support and pathogen resources.

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Lisa and Mark Tatara for listening to my research.
Cross inoculations to observe broad spectrum SAR

Fig. 12: Broad spectrum SAR: B. cinerea and P. irregulare

DISCUSSION

REFERENCES
Abstract:
Systemic acquired resistance (SAR) is a well-characterized defense mechanism in vascular plants where initial exposure to a pathogen induces resistance throughout the plant to subsequent attacks by a wide range of pathogens. A similar SAR-like mechanism has recently been documented in a nonvascular plant, the moss Amblystegium serpens, but it has not been thoroughly characterized. Currently only one pathogen, the oomycete Pythium irregulare, has been shown to trigger SAR in this or any other nonvascular plant. I have observed and characterized the interactions between A. serpens and two other alleged moss pathogens, the ascomycetes Acrosporium sympodiale and Botrytis cinerea. Using the novel culture system that was developed during previous studies in the Hauck lab, I aimed to determine if these fungi can trigger a similar SAR response in the moss. Finally, I planned to determine if the SAR response in moss results in resistance to a range of pathogens or if it is specific to the type of pathogen used in the primary inoculation. Due to the inability to infect A. serpens with these ascomycetes, the SAR response still remains uncharacterized and results in this research were inconclusive.
**Introduction:** A plant’s ability to survive is dependent on how successful it is in its environment with regard to its structure, function, and defense mechanisms. Not only is a defense system critical to a plant’s ability to survive in nature, but it is central to the agricultural economy. In the United States, farmers nationwide spend $4.1 billion on pesticides every year (Agriculture, 2012). Pesticides are costly and are attributed with polluting the natural environment as well as being a danger to humans through groundwater contamination. Therefore, by understanding how a plant’s immune system works, it can give insight to the agricultural community how to protect crops from pathogens in a more effective and environmentally friendly manner. While research into these defense mechanisms is important to the agricultural economy, it is also critical to our understanding of the evolutionary history of pathogen resistance in plants.

Land plants first appeared around 475 million years ago (Campbell *et al.*, 2009). These first plants were nonvascular plants, also known as bryophytes (Fig. 1). The first bryophytes included the liverworts, hornworts, and finally mosses (Fig. 2). Due to their lack of a vascular system for water and sugar transport, these plants were small and grew close to the ground and in moist environments. The next plants to appear included the ferns, which were the first plants to possess a developed vascular system, followed by gymnosperms, the first true seed bearing plants, and angiosperms, the first flowering plants (see Fig. 1). Each evolutionary event brought new structures to facilitate survival through more advanced modes of reproduction, transport, and communication. While the origin of vascular tissue and seeds is known, others, like defense systems, remain unknown.
Bryophytes:

There are 24,700 species of bryophytes that include the hornworts, liverworts, and mosses (Fig. 2). Although, bryophytes do not have a well-developed vascular system, some have rudimentary conducting structures. While bryophytes lack true roots that can absorb water, they do have rhizoids that hold them in the ground. Liverworts are presumed to be the first land plants and are composed of spikes arranged in a circle coming from the gametophyte (Nonvascular, internet). While some liverworts have flattened gametophytes, others are leafy and resemble mosses (Nonvascular, internet). Moss is composed of leaf-like structures that spiral around rhizoids, which act as a source of anchorage for the plant (Nonvascular, internet). Bryophytes have simple modes of water and nutrient transport through their bodies, meaning they lack the lignin-impregnated conducting cells that vascular plants have (Farabee, 2004). As with all plants, bryophytes have a sporophyte (diploid) and gametophyte (haploid) phase; however, in contrast to all higher plants, the prominent phase in bryophytes is the gametophyte (Fig. 3). Bryophyte gametophytes grow close to the ground since they do not have the necessary water conducting cells to permit the development of long stems (Campbell et al., 2009).

The moss genus *Polytrichum* has central conducting cells that developed through convergent evolution (Campbell et al., 2009). These cells are called hydroids and leptoids. Hydroids conduct water and are thin-walled (Fig. 4). They are surrounded by a cell wall component, similar to lignin that enables transport throughout the plant (Water, internet). Leptoids function in the transport of nutrients. Each cell is connected by plasmodesmata, which are small pores that allow flow of cytoplasm between cells. The conservation of
these conductor cells varies dramatically. They are not found in liverworts and only appear some in some moss species (Water, internet). Thus, due to the global lack of vascular tissues, bryophyte gametophytes must live in moist environments and close to the ground for survival. Moist environments also enable their motile sperm to swim to the egg during reproduction.

Because of this lack of nutrient conduction, the sporophyte is small and stays connected to the gametophyte for nutrients. As seen in Fig. 5, the sporophyte is only composed of a foot, which is connected to the parent gametophyte, the stalk, called the seta, the capsule, which holds the spores, and the peristome, which is a structure that releases the spores (Campbell et al., 2009). The sporophytes of some moss and hornworts have stomata, which are pores that function in gas exchange. Stomata are characteristic of vascular plants; thus, this leads to uncertainty regarding the origin of these structures in nonvascular and vascular plants.

Vascular Plants:

The vascular plant system of ferns, gymnosperms, and angiosperms (collectively referred to as 'vascular plants') consists of two main components: the xylem and phloem. The xylem enables the transport of minerals and water throughout a plant through specialized cells called tracheids, while the phloem allows movement of macromolecules, especially sugar (Campbell et al., 2009). The presence of a developed vascular system allows these plants to grow tall and live in varying environments. This vascular system also enables a mechanism for the transport of signals, such as hormones, throughout the plant. This transport will enable one region of the plant to send a signal to another region
in order to induce a response, such as changes in the expression of a certain genes. Among other things, the ability to transport signals is vital in the plants’ defense.

**Plant Defense in vascular plants:**

Interactions between plants and pathogens have resulted in significant coevolution since the time plants first emerged. This constant coevolution has resulted in the development of numerous, well-characterized defense mechanisms in vascular plants. The first line of defense in vascular plants involves physical barriers. Some examples of physical barriers in vascular plants are cell walls (which are also present in nonvascular plants), waxy cuticles on leaves, and corky bark in the stems of some vascular plants. These barriers can act by preventing the invasion of the plant cell by pathogens. However, when the physical barriers are not enough, inducible defense systems can be triggered. Two well-characterized inducible defense mechanisms in vascular plants are the hypersensitive response (HR), which is a rapid and localized response, and the systemic acquired resistance (SAR), which allows increased resistance throughout the plant. These two defense systems are crucial to the plant’s survival in the presence of pathogenic bacteria, fungi, or viruses (Loon et al., 2006).

**The Hypersensitive Response (HR) in Vascular Plants**

HR occurs when the plant recognizes the product of avirulence (avr) gene in the pathogen (Morel and Dangl, 1997). This product triggers a cascade of events that includes activating anti-microbial proteins and enzymes that result in localized necrosis (i.e. death of the plant tissue). An HR can be activated by not only pathogens but
increased exposure to ultraviolet light and physical wounding (Brederode et al., 1991).
In this event, the response is localized and not generally linked with future resistance, and
the main goal is to kill the plant’s own tissue in order to prevent the spread of the
pathogen. Vascular plants may also produce secondary metabolites that act as chemical
defenses. For example, terpenoids are produced by leaves and they act to protect the cell
membranes from high temperature or light damage. Monoterpenoids and
sesquiterpenoids are volatile compounds that protect against fungi or bacteria pathogens
(Freeman and Beattie, 2008).

*The Systemic Acquired Resistance (SAR) Response in Vascular Plants*

SAR occurs through the induced expression of pathogenesis-related (PR) genes
that are activated by a complex pathway that involves the hormones salicylic acid (SA),
jasmonic acid (JA), and ethylene (Loon et al. 2006). For example, when a plant senses a
pathogen, there is a surge in the production of ethylene that plays a key role in regulating
transcription of these PR genes (Ecker and Davis, 1987).

With the influx of ethylene concentrations, the defense enzymes L-phenylalanine
ammonialyase (PAL), 4-coumarate CoA ligase (4-CL), chalcone synthase (CHS), and
hydroxyproline-rich glycoproteins (HRGPs) have increased expression. PAL, 4-CL, and
CHS enable a plant to have increased resistance to later attacks, while HRGPs respond to
cell wall damage and also enable a plant to have increased resistance (Ecker and Davis,
1987). Thus, responses include changes in the cell wall and the production of anti-
microbial compounds (Oliver et al., 2009).
The PR genes in tobacco and tomatoes have been well-characterized (Loon et al., 2006). PR genes have a wide range of defense functions; for example, some PR genes encode chitinases and beta glucanases, which both recognize and destroy fungal cell walls. Others encode peroxidases and oxidases, which both are able to kill host and pathogenic cells (Loon et al., 2006). In tomatoes, for example, PR-7 functions in breaking down the cell wall of pathogens, while PR-9 functions in strengthening the cell wall of the tomato when under attack. PR genes may not be expressed in young plants during development but will later appear during an infection (Loon et al., 2006).

*SAR in vascular plants is broad spectrum*

SAR can be induced by a wide range of pathogens, and once initiated, provides resistance not only to the species that triggered it, but to a wide range of other pathogens. For example, when SAR is activated in cucumber plants, they have resistance to infection by 13 different fungi, bacteria, and viruses (Kessmann et al., 1994). Another example is that tobacco leaves that have been inoculated with the fungus *Thielaviopsis basicola*, show resistance to secondary inoculations with the tobacco necrosis virus and other pathogens (Kessmann et al., 1994).

Homologs of the PR-1 gene have been found in fungi, insects, and vertebrates; however, its function continues to be uncharacterized (Loon et al., 2006). Importantly, the PR-1 proteins have been conserved throughout evolution, which illustrates the importance of systemic defense. Vascular plants, as with other organisms, are successful, in part, because of broad spectrum resistance mechanisms.
Nonvascular plant defense:

While researchers have characterized defense mechanisms in vascular plants, nonvascular plant defense mechanisms have received very little attention. Past experiments with the moss *Physcomitrella patens* showed that nonvascular plants undergo HR when inoculated with the fungus *Botrytis cinerea* or the bacteria *Erwinia carotovora*, which was characterized by localized cell death (Ponce de León et al., 2007). Other research determined that the genome of *P. patens* includes genes that are homologous to defense genes in vascular plants, suggesting the probability of an SAR-like defense (Andersson et al., 2004). Additionally, a study showed that growing *P. patens* protonema tissue, part of the gametophytic phase in moss, in media that contained the hormone SA resulted in the improved resistance to the bacteria, *E. carotovora*, which may suggest a SAR-like mechanism in moss (Andersson et al., 2004). Finally, Oliver et al (2009) showed that a growth of moss in the presence of the hormone JA induces expression of two non-PR genes that play a role in defense (CHS and PAL). All of these studies suggest that moss might have some conserved defense mechanism.

Using a novel culture system, previous research in the Hauck lab discovered an SAR-like response in the moss *Amblystegium serpens* (Winter et al., submitted). A primary inoculation with the oomycete *Pythium irregulare* resulted in nearly complete resistance to secondary inoculations with the same pathogen. They also found that the presence of β-1,3 glucan, a component of the oomycete cell wall, was sufficient for triggering SAR. Thus, despite the lack of a well-developed vascular system, moss appears to be able to transmit signals and induce systemic resistance to future attacks.
Broad spectrum resistance in nonvascular plants

Although a SAR response appears to have occurred in previous nonvascular plant studies, the existence of broad spectrum resistance has yet to be explored. Studies aimed at determining if non-vascular plants have a broad spectrum resistance because this finding could help us determine if the SAR mechanism in vascular and non-vascular plants are analogous. Thus, using a novel method developed in previous experiments, broad spectrum resistance in *A. serpens* was explored using the pathogens *P. irregulare*, *Acrosporium sympodiale*, and *Botrytis cinerea*. *A. serpens* will be exposed to one of the pathogens and then re-exposed to a different type of pathogen at a later time. If *A. serpens* survives without showing signs of necrosis following the secondary inoculation, then it can be assumed that a broad spectrum SAR response occurs in *A. serpens*.

The pathogens used in this study include the oomycete *P. irregulare* and ascomycetes *A. sympodiale* and *B. cinerea*. Oomycetes have a different cell wall composition than ascomycetes. The oomycete cell wall is composed of beta glucans and cellulose, while the ascomycetes have cell walls composed of chitin with little, if any, cellulose (Rossman and Palm, 2006). Since previous studies have shown an interaction between the moss and pathogens, the moss must be able to sense the differences in cell wall composition of the pathogen to initiate a defense response. The moss is able to sense the pathogen associated molecular patterns (PAMPs) on the surface of the pathogen that include differences in cell composition molecules (Boller and Felix, 2009). The ability for one of these three pathogens to induce systemic resistance in the moss to the other type of pathogen would be a positive sign that SAR in moss is broad spectrum, as it is in vascular plants.
Goals and hypothesis:

The goal of the proposed research is to determine if the SAR response in moss is broad spectrum, as it is in vascular plants. Using three different pathogens, exposure to one pathogen should initiate future resistance to the other pathogens in *A. serpens* if SAR in moss is broad spectrum. If *A. serpens* does not show signs of necrosis after a secondary inoculation using a different pathogen from the first, then broad spectrum SAR occurs in *A. serpens*. I hypothesize that SAR in moss is broad spectrum.

Methods and Materials

Moss Maintenance

*A. serpens* was maintained on Murashige and Skoog Salts (MS) 2.165 g/500 mL in agar in 5.2 g/L of Agargel. The agar was used at pH of 5.8. The medium was autoclaved and poured in separate petri dishes, covered, and left to solidify overnight. The moss was maintained on a 16 hr/day light-dark schedule in 25°C and subcultured periodically to continue growth.

Pathogen Maintenance

I used the pathogens *B. cinerea*, *P. irregulare*, and *A. sympodiale* to infect *A. serpens*. *B. cinerea* was maintained in the dark on V8 medium that was composed of 36 ml/L of V8 juice, 2 g/L of CaCO3, and 20 g/L of bacteria agar. Eight day old cultures were used for inoculation. Two-day-old *P. irregulare* and *A. sympodiale* were maintained on potato dextrose agar (PDA), which was made of 39 g/L of PDA agar mix. All of the media were autoclaved, poured into separate petri dishes, and left to solidify overnight.
The pathogens were subcultured periodically to allow continued growth and to initiate growing fronts that could be used for inoculations.

Inoculations

Inoculations were done by taking small cubes (approximately 3 mm³) of agar containing fungal mycelium from the growing front of the fungus and placing them on an end of the moss. The cubes of agar containing the pathogen were removed in a laminar flow hood using sterile techniques.

Verification that pathogens can infect A. serpens

After inoculating A. serpens with a pathogen, I expected to see symptoms of the infection, which would include necrosis and chlorosis, which can be observed without the aid of a microscope (Ponce de León et al., 2007). Light microscopy was also done following lactophenol trypan blue staining, which specifically stained the fungal mycelium.

Testing for SAR

Using the novel system developed previously in the Hauck lab, I tested for an SAR-like broad spectrum response in A. serpens. For each experiment, I used a special divided petri dish that was arranged with two of the four quadrants filled with MS media. I placed a piece of A. serpens on the plate so that one end was on the media and the other end was overhanging the adjacent empty quadrant. I then inoculated the overhanging end with one of the pathogens or the control (water). I then cut the moss in half at 16, 24, or
32 hours after the primary inoculation. I then transferred the un-inoculated end to a new plate and secondarily infected this piece with the same pathogen. If the fungus in the secondary inoculation of the experimental group failed to infect the moss, then SAR was induced (Fig. 6).

Testing for the induction of SAR by chitosan in A. serpens

Using the previously described experimental setup, I worked to determine if chitosan, a component of the fungal cell wall, could trigger SAR against each of the three pathogens being studied. I dropped 3 µl of 1 mg/ml of chitosan (in water) or water on the moss 24 hours prior to inoculation with pathogen. Failure of the fungal pathogen to infect the moss following chitosan application suggested that the chitosan induced SAR.

Testing for Broad Spectrum SAR

Using previously discussed inoculation method, A. serpens was inoculated on one end with one pathogen 16, 24, or 32 hours prior to secondary inoculation with one of the other pathogens on the unexposed end of the moss (see Fig. 7). If the fungus in the secondary inoculation failed to infect the moss, then SAR was induced.

Modifying the method of inoculation

When the original method of inoculating A. serpens did not seem to work due to the lack of response in the moss with each pathogens, I devised other ways to facilitate the infection of the moss by the pathogens. First, I tried to physically wound the moss prior to inoculation. I tried several different methods of wounding the moss, including
gentle tapping with a glass rod, lightly brushing with a razor blade, and tapping with a dissecting needle. I believed the physical wounds would provide an entry point for the pathogen.

Additionally, I tried inoculating the moss with isolated spores of *B. cinerea*. After failure to initiate a response with *B. cinerea* mycelia, I prepared spore suspensions from 14 day old cultures. The spore cultures were diluted to a concentration of $2 \times 10^5$ spores/ml, which were placed directly on one end of the moss. The spore cultures were collected from a 14 day old culture using sterile forceps to scrape the culture’s growth front. The spores were collected in a test tube of sterile, distilled water. A previous study used a concentration of $5 \times 10^4$ spores/ml in 0.05% K2HPO4. (Hou and Chen, 2003). Thus, the number of spores was then counted under a light microscope using a haemocytometer in order to get a final concentration of $2 \times 10^5$ spores/ml. Before inoculating the moss with 3 μl, the tubes holding the spore cultures were vortexed.

**Analysis**

Large-scale necrosis was observed with the naked eye. Using the light microscope, cytoplasmic shrinkage could be observed. Images were taken using a compound microscope to compare the moss before and after inoculations to determine if the pathogens could infect the moss. These images were compared to images taken of the control.
**Results:**

*Inoculation with B. cinerea*

Exposure of *A. serpens* with *B. cinerea* induced little response. Necrosis was not apparent from the naked eye as well as under magnification using the light microscope. Under 10x magnification using the compound microscope, fungal growth was apparent (Fig. 9). Viewing the moss under 20x magnification, possible cytoplasmic shrinkage was seen in a localized region (Fig. 10 and Fig. 12). However, the reliability of infection was poor.

*Inoculation with P. irregulare*

Exposure of *P. irregulare* to *A. serpens* did not show any signs of necrosis. After repeating the plug inoculation method multiple times, all inoculations resembled the control (Fig. 8).

*Inoculations with A. sympodiale*

*A. sympodiale* did not grow successfully in culture. Thus, in every trial, the moss resembled the control (Fig. 8). I tried culturing *A. sympodiale* multiple times.

*Modified inoculation trials*

Physical wounding of the moss prior to inoculation or using isolated spore inoculations did not improve success of the infection; once again, the moss post-inoculation appeared similar to the control (Fig. 8). Isolated spore inoculations were performed in multiple trials.
SAR response to chitosan

Inoculating A. serpens with chitosan initially with a second inoculation of B. cinerea showed no necrosis or cytoplasmic shrinkage. The moss resembled the control (Fig. 8).

SAR response to B. cinerea

There was no apparent necrosis or cytoplasmic shrinkage (Fig. 11). However, control also showed no necrosis or cytoplasmic shrinkage.

Cross inoculations to observe broad spectrum SAR

Cross inoculations between B. cinerea and P. irregulare showed no apparent differences from control (Fig. 8). Cytoplasmic shrinkage was seen in a primary and secondary inoculation of B. cinerea and P. irregulare, respectively, but the lack of appearance of fungal growth suggests that the cytoplasmic shrinkage was not due to the inoculations (Fig. 12). Again, the controls did not show symptoms of infection.

Discussion:

SAR is a critical component of pathogen and viral defense in vascular plants for the ability to induce not only localized but whole plant defense through the expression of pathogen-resistance genes. A SAR-like effect has been discovered in A. serpens previously using P. irregulare (Winter et al., submitted), and studies by Oliver et al. (2009) found PR gene induction in a species of moss. Thus, SAR has been shown to be present in mosses.
Due to the inability to reliably infect *A. serpens* with any of the pathogens, an SAR response could not be induced in this study; consequently, the characterization of SAR in *A. serpens* remains inconclusive. While secondary inoculations showed no response to the primary pathogen inoculation, the lack of necrosis or large-scale cytoplasmic shrinkage suggests that the pathogens were ineffective in the primary and secondary inoculations to affect the plant. Besides minute changes in cytoplasmic shrinkage after inoculations with *B. cinerea*, the moss did not differ from un-inoculated specimens (Fig. 8).

The inability to infect the moss could be due to the vigor of the fungal cultures. While *P. irregulare* and *B. cinerea* grew in cultures, they may have changed genetically, a phenomena called somaclonal variation, or became contaminated with another pathogen that may explain their ineffectiveness. Additionally, the *B. cinerea* spore concentration may need to be more concentrated. A previous study by Ponce de León *et al.* (2007) found that *B. cinerea* was able to infect the moss *P. patens*, which may suggest that *B. cinerea* may not be pathogenic to the strain of *A. serpens* used in this study. However, Winter *et al.* were able to infect this same strain of *A. serpens* with *P. irregulare* in a previous study.

Because *P. irregulare* and *B. cinerea* have been shown to be able to infect *A. serpens* (Bowman, 2011; Ponce de León *et al.*, 2007), this study should be repeated using *P. irregulare* and *B. cinerea* and possibly the bacterium *Erwinia carotovora* that was used in previous experiments with *P. patens* and caused HR (Ponce de León *et al.*, 2007). Additionally, *Acrosporium* should be used since its effects were not seen in this study due to its inability to grow in culture.
The presence of broad spectrum SAR in moss should be continued to be explored in order to have a better understanding of the origins of defense in plants, as well, to determine if moss can be used in research as a model for SAR in vascular plants. Using moss as a model specimen will enable future studies on fungicide penetrance in a model that is easy to maintain and experiment on than a vascular plant.
References:


Nonvascular plants are relatively unspecialized, but successful in many terrestrial environments [Internet]. [cited 1 Mar 2013]. MHEE. Available from http://www.mhhe.com/biosci/genbio/tlw3/eBridge/Chp16/16_2.pdf


Water relations: Conducting structures [Internet]. [cited 1 Mar 2013]. MTU. Available from http://www.bryoecol.mtu.edu/chapters/7-1Conductstruc.pdf

**Figure 1: Evolutionary history of land plants.** The first land plants appeared around 475 million years ago. Vascular plants diverged from non-vascular plants approximately 425 million years ago. This figure was adapted from The evolution of plants [Internet]. 28 June 2011 [cited 5 March 2013]. Antranik.org. Available from http://antranik.org/the-evolution-of-plants/
Figure 2: Examples of bryophytes. All bryophytes lack a vascular system, seeds, and flowers. Available from http://www.cfkeep.org/html/phpThumb.php?src=/uploads/bryophytes.gif&aoe=1&w=
Figure 3: Moss life cycle. Bryophytes, unlike other major groups of plants, have a more prominent gametophytic stage than the diploid sporophytic stage, due to the lack of vascular tissue and motile sperm. Due to the evolution of vascular tissue, spores, and seeds, in upper-level plants that include ferns, gymnosperms, and angiosperms, the sporophyte stage is dominant because of its novel independence.

Available from http://www.cavehill.uwi.edu/bio_courses/bl14apl/images_bryos/moss_life_cycle.jpeg
Figure 4: **Moss conducting system.** The leptoids and hydroids are primitive and rudimentary conducting cells in moss. The seta is the stalk of the sporophyte that connects the parent gametophyte to the capsule, which holds the spores. The parenchyma is made of cortical cells that make up the inner portion of the stem, while stereid cells provide structural support.

Available from http://palaeos.com/plants/images/MossSetaXS.gif
Figure 5: **Moss gametophyte and sporophyte.** The most prominent part of a moss plant is the green, grass-like gametophyte. The sporophyte is simply a short stalk and capsule where spores are produced. Available from [http://www.biosci.ohio-state.edu/~plantbio/osu_pcmb/pcmb_lab_resources/images/pcmb102/algae_mosses/moss_structure.jpg](http://www.biosci.ohio-state.edu/~plantbio/osu_pcmb/pcmb_lab_resources/images/pcmb102/algae_mosses/moss_structure.jpg)
Figure 6: Testing for SAR in *A. serpens*. One end of the moss was inoculated with a pathogen (B) or the control (W, water). The shaded regions of the plate represent the presence of medium; thus, the inoculated end of the moss overhung an empty sector of the plate. The moss was then cut at 16, 24, or 32h, and the unexposed end was transferred to another plate. The moss was then treated with the same pathogen (B). Lack of necrosis suggested SAR.
Figure 7: Broad spectrum SAR with *B. cinerea* and *P. irregulare*. A strand of the moss was inoculated with one of the pathogens (B or P). After 16, 24, or 32h, the moss was cut, and the unexposed end was transferred to another petri dish. The moss was then inoculated with a different pathogen (B or P, whichever was not used for the primary inoculation). Lack of necrosis suggested broad spectrum SAR.
Figure 8: Uninoculated A. serpens. All cells show full chloroplast and cytoplasmic expansion. 20x magnification was used.
Figure 9: *B. cinerea* branching throughout *A. serpens*. Fungi growth is depicted by the arrows. 10x magnification was used.
Figure 10: A. *serpens* leaf seven days post inoculation with *B. cinerea*. Symptoms of infection include possible localized chloroplast and cytoplasmic shrinkage as depicted by the arrow. 20x magnification was used.
Figure 11: Testing for SAR using inoculations of B. cinerea. There appears to be no cytoplasmic shrinkage or necrosis after primary and secondary inoculations of B. cinerea as well as a lack of fungal growth, which shows the failure for the fungus to even grow on the moss. 20x magnification was used.
Figure 12: Broad Spectrum SAR using *B. cinerea* and *P. irregulare*. There appears to be no necrosis, however cytoplasmic shrinkage is occurring, as depicted by the arrow, after inoculating the moss initially with *B. cinerea* and secondly with *P. irregulare*. Yet, the lack of fugal growth suggests that the cytoplasmic shrinkage was not due to pathogen inoculations. 20x magnification was used.