5-2022

Investigation Into the Responses of Physcomitrella patens to Herbivory and the Influence of Light on the Production of Defense Compounds

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BUTLER UNIVERSITY HONORS PROGRAM

Honors Thesis Certification

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Thesis title Investigation into the Responses of Physcomitrella patens to Herbivory and the Influence of Light on the Production of Defense Compounds

Intended date of commencement May 06, 2022

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Date

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Date

Investigation Into the Responses of *Physcomitrella patens* to Herbivory and the Influence of Light on the Production of Defense Compounds

A Thesis

Presented to the Department of Biological Sciences

College of Liberal Arts and Sciences

and

The Honors Program

of

Butler University

In Partial Fulfillment

of the Requirements for Graduation Honors

Matthew Thomas Morris

May 06, 2022

Acknowledgments

I would like to thank Dr. Philip Villani for the guidance and resources he bestowed upon me. Without his help, I would not have been able to complete this research. I would also like to thank the Butler Biology Department for providing me with the understanding to take on such a task. I am also grateful for my friend, Ammar Razzaq, who helped me with a sentence.

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Abstract

Plant defense mechanisms are well studied in many agriculturally relevant crops such as corn and tomatoes. Though less studied, the more ancestral nonvascular plants may be able to provide insights into the origin and working of modern plant defense systems. In this study, the bryophyte *Physcomitrella patens* was researched and the role of the jasmonic acid pathway in response to herbivory was investigated. Additionally, the impact of light intensity on the efficacy of this pathway was to be determined. After exposing the moss to various chemical elicitors and mechanical wounding, the activity of proteins produced in the JA pathway was measured. In *P. patens*, no definitive conclusions could be drawn regarding the role of JA in the defense against herbivory. Due to a lack of results from the initial experiments, the light experiments were not performed.

Introduction

Defense Mechanisms in Plants

Throughout the lifetime of a plant, it will encounter various challenges in its environment that it needs to overcome. Virtually every part of the plant has adapted to exist and interact with organisms and their environment. They produce flowers to attract pollinators, wide leaves to capture more sunlight, and durable seeds that can withstand freezing weather or seeds that even rely on blazing wildfires as a trigger for germination (Riveiro *et al., 2019*). When competing with other plants, they can respond in many ways. Growing taller or growing more broad leaves allows them to obtain more sunlight than their neighbors while growing deeper, wider roots allow them to get more water or nutrients from the soil. Some species of plants reduce competition with others by emitting chemicals to inhibit the growth of nearby plants, called allelopathy, which prevents them from efficiently gathering resources (Mauseth, 2016). While plants must compete with other plants for space, light, and resources, they aren't the only organisms to worry about. They will need to defend themselves against predators as big as cows and pathogens as small as viruses (Gurevitch *et al.,* 2002).

While many other organisms interact with plants, the most detrimental interaction plants can have with these organisms is herbivory, or the consumption of the plant by another organism. Plants have been in the presence of herbivores for hundreds of millions of years, resulting in the evolution and development of complex defense mechanisms to protect against the many different types of herbivores. There are two main categories of defenses: constitutive and inducible. Constitutive defenses are always present and act as physical or chemical barriers that prevent or deter animals from eating or destroying them (Taiz *et al.,* 2014). For example, some plants have large thorns or spines that protect against grazing from larger animals such as cows or horses. Another example, stinging nettle (*Urtica dioica*), is a frustrating example of constitutive defense that many people may be familiar with. Even a brief encounter with this plant will leave a lasting impression due to its unique defense system. The leaves of the stinging nettle are covered in small needle-like hairs called trichomes that are filled with chemicals like histamine, acetylcholine, and formic acid which cause a burning rash. When an organism is unlucky enough to brush against the leaves, the trichomes are embedded in the skin and break off, releasing that cocktail under the skin (Fu *et al.,* 2006). Trichomes are not only for large herbivores or unsuspecting passersby. For smaller organisms like insects, the density and shape of trichomes prevent them from holding onto the plants, while some glandular trichomes may burst open upon contact and release strong-smelling, bitter-tasting compounds that deter the insect herbivore.

Constitutive compounds can also be found inside the plant tissue itself. Chemicals that are stored in plant cells are released when the leaf is broken or chewed, creating a strong taste and indicating that the plant may be toxic to consume. The concentrations of chemical compounds in plants are thought to follow what is known as the optimal defense hypothesis. This is the idea that because the plant only has a limited number of constitutive compounds, they will be stored in parts of the plant that will maximize fitness. This means that younger leaves, which are more valuable than older leaves, will have greater concentrations of these chemical defenses. These younger leaves will therefore be stronger tasting and more likely to prevent herbivory (Massad *et al.,* 2014).

Ironically, the same chemicals that were produced to deter predators are exactly what attracts us to them. Caffeine in coffee, for example, is a wonderful example of a constitutive compound. It can be found in the fruit of the coffee plant (*Coffea arabica*) and benefits the plant in two ways: a chemical defense and an allelopathic offense. Found in the fruit of the coffee plant, caffeine is a natural pesticide as it is toxic to many insects (Nathanson, 1984). This protects the plant from herbivory while it is still on the tree. When that fruit eventually falls, the caffeine acts as an allelopathic chemical, shunting the growth of nearby plants and allowing the new coffee plant plenty of room to grow (Silva *et al.,* 2013). Additionally, many of the herbs that we cook with daily, like garlic, ginger, or basil, are used because of these defense compounds. The strong-tasting chemicals like allicin, gingerol, and eugenol that normally deter herbivores are the same that give our food delicious and complex flavor (McGinley, 2006).

The other main type of defense, inducible defenses, are the physiological changes and chemicals produced when a plant experiences damage or stress like that of an attacking herbivore (Taiz *et al.,* 2014). Inducible defenses can be beneficial over constitutive defenses for two main reasons. First, it is energetically expensive to maintain secondary metabolite concentrations at optimal levels and create defensive structures even when not being attacked. Second, if a defense is always present it is much easier for an organism to adapt to and eventually overcome those defenses. Having a more flexible and species-specific response enables the plant to defend itself better (Taiz, *et al.,* 2014). With this type of defense, plants must be able to detect the attack, then activate the molecular pathway that sends signals elsewhere in the plant to alter gene expression or metabolism to produce defense compounds.

When herbivory occurs, two things happen: mechanical damage and the introduction of oral secretions. When a hungry caterpillar takes a bite of a leaf, the subsequent tearing and destruction set off alarms in the plant, though that by itself may not be enough. The plant must be able to differentiate between abiotic, or environmental, factors like strong wind or hail, and biotic factors like an insect. Insects make up a major portion of herbivores and can be separated into three main groups. First are the chewing insects, such as the caterpillar previously mentioned. These insects cause significant damage to plants by tearing and consuming large amounts of plant tissue. Another group is the phloem feeders. Including aphids and leafhoppers, this group pierces the outer layers of the plant tissue, including the epidermis and sclerenchyma, with sharp mouthpieces called stylets and drink up the nutrients from the phloem, or vascular system, of the plant (Figure 1). While they do not cause much physical damage by themselves, they can also act as vectors, injecting pathogens deep into plants. The final group of insect herbivores is the cell-content feeders, such as mites, which cause an intermediate amount of damage to cells and also threaten to introduce pathogens (Taiz *et al.,* 2014). A common aspect of all of these types of insect herbivores is that they all release oral secretions when feeding. Within the oral secretions are species-specific molecules, called elicitors, that can be detected by the plant and distinguish it from an abiotic factor. While many organisms, including pathogens, can produce elicitors, insect herbivores have recently been given the name herbivore-associated molecular patterns (HAMPs), which are similarly detected by the plant (Basu *et al.,* 2018). Examples of common HAMPs used in research are caeliferins, volicitins, and chitosan, which can be extracted from grasshoppers, caterpillars, and fungi which possess the ability to deacetylate the main structural components of insect exoskeletons, respectively (Taiz *et al.,* 2014; Yin *et al.,* 2016).

After mechanical damage occurs and elicitors are detected, plants start to produce signaling molecules such as H_2O_2, Nitric Oxide (NO), and other secondary metabolites. These alert the plant to begin the production of local and systemic defense metabolites (Taiz *et al.,* 2014). These can be used to deter herbivores, begin the healing process, or even signal predators of the herbivore. An interesting example of this can be found in the wild tobacco plant (*Nicotiana attenuata*). Both the elicitors within the saliva of the tobacco hornworm (*Manduca* *sexta*) and even the rhythm of the caterpillar eating alert the plant to what species is attacking it. In response, the plant releases hexenol, a secondary metabolite that alerts nearby predators like the big-eyed bug (*Geocoris spp.*). The big-eyed bug then looks for the plant and preys on the eggs and young caterpillars, protecting the plant (Allman & Baldwin, 2010). Another example is freshly cut grass. That pleasant smell is actually the plant sending out distress signals to nearby plants, signaling them to prepare for a possible attack (Wei *et al.*, 2007).

Not all defenses fit perfectly into the categories of inducible or constitutive, however. For example, plant defensins are small, basic antimicrobial peptides that are characterized by their three-dimensional folding pattern and eight cysteines linked by disulfide bridges (Thomma *et al.,* 2002). Defensins are found in a wide variety of organisms, including humans, insects, and plants. Regardless of the organism, all defensins play a role in the immune system, targeting bacteria and viruses. (Jenssen *et al.,* 2006). These small peptides have been found to be both inducible and constitutive. This is mainly due to the variety in origin and function. (Khan *et al.,* 2019).

Plant Signaling and Hormones

In order to organize local and systemic events inside the plant and send signals outside the plant, communication must occur. To accomplish this, plants use hormones, much like humans. Hormones are chemical messengers that are produced in one cell, travel to another cell, cause molecular changes within that cell by binding to a receptor, and activate a signal cascade, resulting in transcriptional or post-transcriptional modifications, and ultimately, developmental or physiological responses. Also, much like humans, plants use hormones in very low concentrations, meaning it does not take a lot of damage before the whole plant starts to respond (Taiz *et al.,* 2014).

The development and survival of plants are governed by nine major groups of hormones including auxins, gibberellins, jasmonates, and salicylic acid (Figure 2). Auxin is vital for plant growth and is the main hormone that influences a plant's tropism, or movement. The Cholodny-Went Hypothesis dictates that tropisms within plants are due to unequal distributions of auxin within the plant (Taiz *et al.,* 2014). In a plant that has fallen over, auxin is transported on the lower side causing faster growth on the bottom, but not the top, correcting the orientation of the plant (Mauseth, 2016; Figure 3). Gibberellins are a large class of tetracyclic compounds that contribute to stem growth, germination, and fruit development. Commercially, G_3, a gibberellin, is used to increase the length of grape stems, allowing for greater separation between bunches and preventing bunch rot. G_3 also works to increase the size of the berries, increasing both their overall survivability and marketability (Jadhav *et al.,* 2020; Dokoozlian & Peacock, 2001).

When it comes to the defense systems of plants, salicylic acid (SA) and jasmonic acid (JA) are the two most crucial signaling hormones. SA is typically activated when the plant encounters microbial pathogens but can also be activated by the stylet penetration of phloem feeders. A typical response associated with SA signaling is the hypersensitive response (HR) in which the cells in proximity to the infection begin apoptosis, or programmed cell death. By initiating this response and killing off infected cells, the plant can limit the spread of the pathogens and possibly save the plant (Ballaré, 2014). Along with this response is the production of other molecules that trigger a systemic defense to protect against the spread of the pathogen to other parts of the plant.

JA, on the other hand, is the main pathway associated with damage from abiotic and other biotic stresses. Upon mechanical damage to a plant and exposure to elicitors, prosystemin, a precursor molecule, is produced by the damaged leaves and hydrolyzed into systemin.

Systemin then leaves the wounded parts of the plant and binds to a receptor on the plasma membrane and begins a complex signal cascade. Once systemin binds to the receptor, it becomes phosphorylated and activates phospholipase A2, a protein that will activate a mitogen-activated-protein kinase (MAPK) cascade to initiate JA synthesis. The MAPK cascade triggers lipoxygenase in the chloroplast to convert linolenic acid into 13-Hydroperoxy linolenic acid. This molecule is then cyclized into 12-oxo-Phytodienoic acid (OPDA), then transported to the peroxisome. In the peroxisome, OPDA is reduced and β-oxidized into JA, and JA is transported throughout the plant. By itself, JA represses growth in plants, allowing them to transfer that energy to defense and increase their chance of survival (Aldridge *et al.,* 1971). To activate many of the defense pathways, JA must conjugate with other molecules such as the amino acid isoleucine. This occurs with the help of jasmonic acid resistance proteins. The resulting jasmonic acid-isoleucine complex (JA-Ile) can then continue to complex with the SCF^{COI1} ubiquitin-ligase complex (Xu *et al.,* 2002). The $SCF^{COI1\text{-}JA\text{-}Ile}$ complex then binds to a family of proteins called the Jasmonate Zim-Domain (JAZ) protein family. These proteins work to repress the MYC2 transcription factor when JA levels are low. Because MYC2 switches on many of the defense responses of the JA pathway, repressing it while JA concentrations are low prevents the unnecessary wasting of energy. The binding of the $SCF^{COI1\text{-}JA\text{-}Ile}$ complex to the JAZ repressor removes it and allows MYC2 to initiate transcription. The complex is then ubiquitinated and degraded by proteasomes (Wasternack & Hause, 2013). Similar mechanisms of JA and other conjugated forms like methyl jasmonate (MeJA) lead to the activation of transcription factor families, like MYCs, and the production of various compounds such as proteinase inhibitors (PI), lipoxygenases (LOX), polyphenol oxidases (PPO), and peroxidases (POD) (Ballaré, 2014). In addition to regulating responses to environmental stress, JA also plays a role in several growth and developmental processes, such as seed and flower development and seed germination (Wasternack & Hause, 2013).

PIs, once produced, travel to the site of damage where an herbivore is consuming tissue, and are consumed. Inside the gut of the herbivore, the PIs inhibit the insect's proteases and prevent it from obtaining resources from the plant tissue. This results in nutrient deficiency that stunts the growth and development of the herbivore (Howe & Jander, 2008). Chemicals like PIs that cause adverse effects or deter feeding from animals or insects are given the name antifeedants. Unfortunately for the plant, many insects have developed their own inhibitors that decrease the effectiveness of plant PIs (Bayés *et al.,* 2005; Giri *et al.,* 1998). Similarly, PPOs, PODs, and LOXs also reduce the nutritive value of the tissue being consumed, though through different mechanisms. Both PPOs and PODs are stored in the chloroplasts, away from phenolic molecules, which they react with. When plant organization is disturbed, as it would be during herbivory, the PPOs and PODs react with the phenolics and produce quinones. These quinones go on to alkylate lysine, histidine, and cysteine side chains in proteins, ultimately lowering their nutritional quality (Constabel *et al.,* 1995; War *et al.,* 2012). Less is known about the exact mechanisms of LOXs, except that they lead to the production of lipid peroxidases and also lower the nutritional value of the tissue being consumed (Duffey & Felton*,* 1991).

Plants and Light

All of these defensive responses require a significant amount of energy and nutrients. Plants are photoautotrophs, meaning they derive their energy from the sun and use inorganic carbon, like CO_2 to produce organic materials. Photosynthesis, the process used by plants to create the sugars necessary for energy production, occurs in the chloroplasts of plants.

Photosynthesis can be divided into two main processes: light-dependent and light-independent reactions. The light-dependent reactions occur within membranous stacks, called thylakoids. Proteins called photosystems I and II span the width of the membrane and use energy from light to trigger the movement of electrons from water to create a reducing agent called nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen. The movement of these electrons also creates a proton gradient which is then used to generate adenosine triphosphate (ATP). During the light-independent reactions, those NADPH and ATP molecules are then in the conversion of CO_2 to a glucose precursor, called glyceraldehyde 3-phosphate (G3P). This molecule is then transported out of the chloroplast and converted into glucose. This glucose is subsequently used to create energy for the plant to use through cellular respiration (Mauseth, 2016; Taiz *et al.,* 2014).

Because a plant requires light to create the food necessary for energy, the amount of light it receives must be taken into account when allocating resources. If a plant is in an environment with low light intensity, it may need to allocate resources away from defense and put them towards getting more light. Everything in plants is governed by the number of resources they obtain. If a plant is competing with a neighbor for sunlight, it may need to allocate more resources for growth than defense. Alternatively, if the plant is fighting a pathogen or a chewing insect, it may have to sacrifice some of those resources for defense. This is why hormones such as JA are important. Once an attack signal has been received, it halts growth and redirects resource allocation towards defense.

Bryophytes

The land plants, or embryophyta, are divided into two main groups: vascular and nonvascular plants. Vascular plants make up the majority of plants you see in your day-to-day life and include trees, ferns, and flowering plants. They are considered “vascular” because of the presence of the lignified tissue, the xylem, which carries water and minerals throughout the plant. Lignified tissue contains large amounts of lignin, a class of polymers that strengthens the tissues of plants and gives them a woody appearance. Additionally, they have the phloem, which is a non-lignified tissue used to transport nutrients and products of photosynthesis (Mauseth, 2016). This vascular tissue allows for the transport of nutrients throughout the organism, allowing vascular plants to grow much larger than their nonvascular counterparts. Also, because of their vascular system, these plants are considered to have true leaves, stems, and roots.

The nonvascular plants, also known as bryophytes, are made up of mosses, liverworts, and hornworts. They lack true leaves, stems, and roots, due to the lack of a vascular system and are instead made up of much simpler tissues. The roots they do have are called rhizoids, which lack the absorptive properties of true roots. They are trichome-like in structure and act to merely hold the rest of the bryophyte in place. Phylogenetic data suggests that bryophytes are far more ancient than vascular plants, which helps explain why bryophytes have such simpler tissues in comparison (Taiz *et al.,* 2014).

Vascular and nonvascular plants also differ in their reproductive systems. All plants cycle between principal generation phases. This includes a haploid gametophyte phase and a diploid sporophyte phase. Vascular plants spend the majority of their lives in the sporophyte phase. Here, the reproduction of vascular plants differs. Ferns, which belong to a group called pteridophytes, produce haploid spores through meiosis and release them into the environment. In gymnosperms (trees) and angiosperms (flowering plants), the female gametophyte is formed within the ovule, the structure that gives rise to the female reproductive cells and remains there. The male gametophyte, pollen, leaves another plant and forms a zygote with the female gametophyte, producing seeds (Holsinger, 2000).

Mosses, such as the organism I worked with, *Physcomitrella patens*, and other bryophytes spend the majority of their lives in the gametophyte phase (Figure 3). This means that the majority of moss you see is a haploid male or female. During reproduction, male gametophytes produce a structure called an antheridial head, which produces sperm through mitosis. When it comes in contact with water, a medium for the sperm to travel in, it bursts and releases the sperm. That sperm then follows the chemical signals released by the archegonial head, the structure produced by the female gametophyte which houses the egg. Once the sperm and egg fuse, the diploid zygote begins dividing and producing a sporophyte. The sporophyte grows from the gametophyte on a long stalk called a seta, at the end of which lies a single sporangium. The sporangium, through meiosis, produces many haploid spores, which are released into the environment once they mature. These haploid spores divide and grow into an intermediate stage called a protonema, which then continues to divide until a fully grown male or female gametophyte is produced and the cycle is completed (Mauseth, 2016). This research will be conducted on the gametophyte stage of the bryophyte *P. patens*. Because this moss was the first to be fully sequenced and because it is easy to grow and propagate, it is widely used and easily manipulated, leading to its designation as a model organism.

Significance

Much research has been done regarding the defense responses of agricultural plants such as tomatoes and corn due to their high economic importance. Due to this, research into the mechanisms of plant herbivory response and how to better protect these plants using these mechanisms is valuable. On the other hand, bryophytes are less researched, but their evolutionary relevance may provide an interesting insight into the defenses of modern-day agricultural plants. Mosses are far more ancient than modern agricultural plants and face the same environmental or organismal pressures. By looking into how they can respond to herbivory, mosses and modern plants can be compared and information regarding the evolution of herbivorous responses could be compared. Previous research on tomato plants has revealed that exposure to herbivory leads to the initiation of several defense responses through the JA pathway (Reinbothe *et al.,* 1994). There is little information, however, on how mosses respond to herbivory, or if they even can respond to herbivory (Farmer *et al., 1992*). Other plants are capable of it and mosses have been around for much longer, so we can hypothesize that they can detect and respond to attacks from herbivores. Additionally, research has been conducted on the response of mosses when exposed to pathogens. This previous research suggests that the herbivory response of mosses is influenced by the intensity of light that it has access to before and during exposure (Griebel & Zeier*,* 2008). However, the question remains: how does the amount of light acquired by the moss, or light intensity, affect the magnitude of this response?

Thesis Hypothesis

Little research exists looking at the defense responses of bryophytes. Because of this, I hope to gain some insight as to how the jasmonic acid pathway is used in the defense responses of the common model organism, *P. patens*. The goal of my research is twofold. First, I will be looking at whether or not *P. patens* is capable of detecting and responding to herbivory. Second, I will investigate how light intensity may impact the various defense responses to herbivory. Using the researched response to herbivory in other plants as a guide, I will look into the various molecular responses of the mosses and attempt to measure the production of PPOs, LOXs, PODs, and PIs. To investigate the impact of light, the mosses will be exposed to varying levels of light (full darkness, shade, full light) then mechanical damage and chemical elicitors will be used to mimic an attack from an herbivore. The production of the same metabolites will be measured and compared between environmental conditions.

I hypothesize that bryophytes, because they are ancestral to modern vascular plants, do possess the ability to detect and respond to herbivory. I predict that when exposed to mechanical damage or known chemical elicitors, the concentration of PPOs, LOXs, PODs, and PIs will increase, as per the JA pathway. Additionally, I hypothesize that, due to the reliance of photosynthetic organisms on light, altering the light levels bryophytes receive will impact their ability to mount a defensive response to an herbivore attack. I predict that reducing the amount of light the plants receive will result in the decreased or delayed production of the same secondary metabolites.

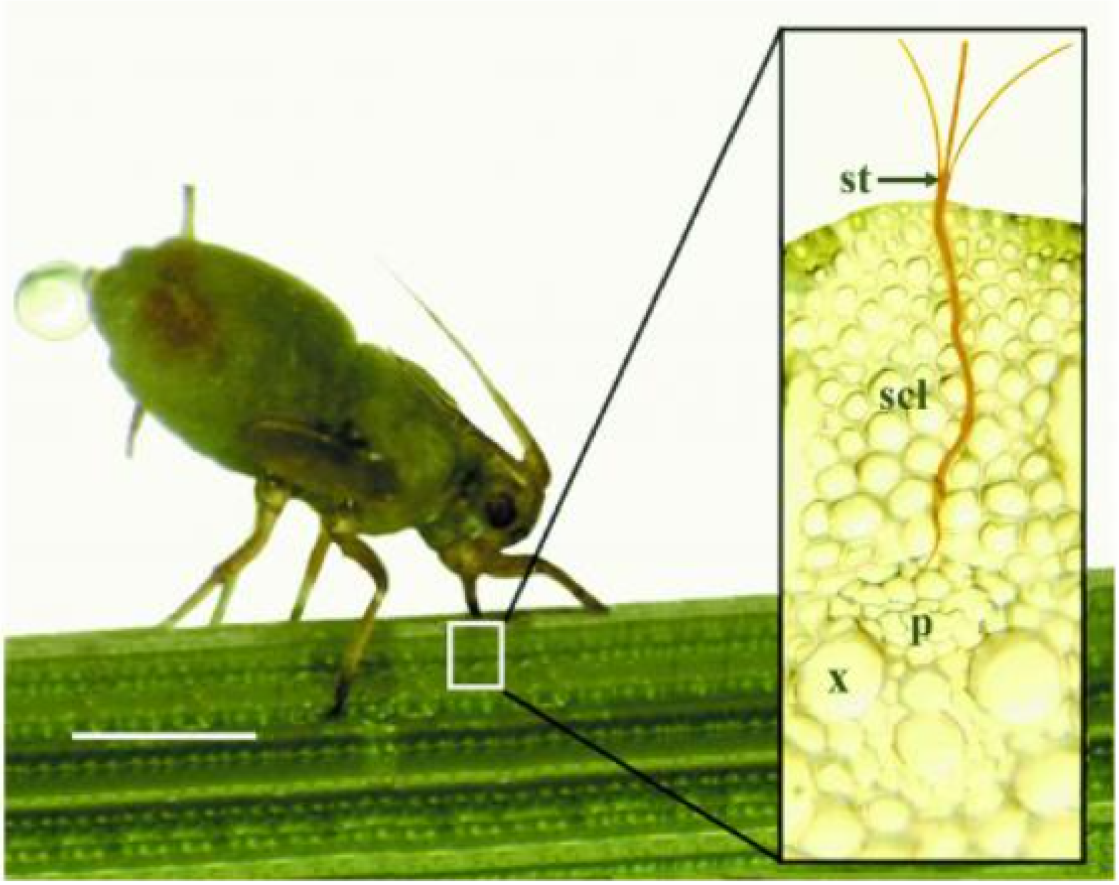
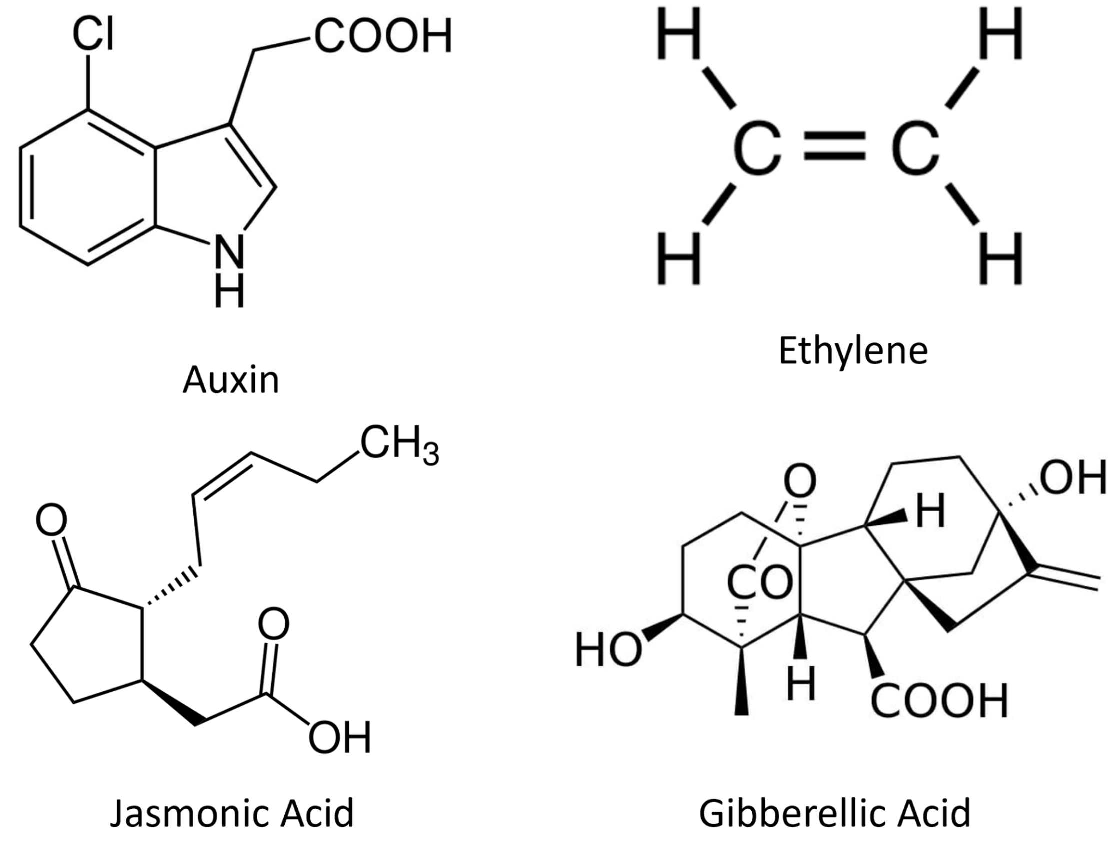


Figure 1. A feeding aphid with stylet embedded in the phloem of a plant; st, stylet; scl, sclerenchyma; p, phloem; x, xylem (Fischer, 2018).



Auxin

Ethylene

Jasmonic Acid

Gibberellic Acid

Figure 2. Chemical structures of four major plant hormones.

Figure 3. Gravitropism as a result of the plant hormone Auxin (Martin Shields, 2018).

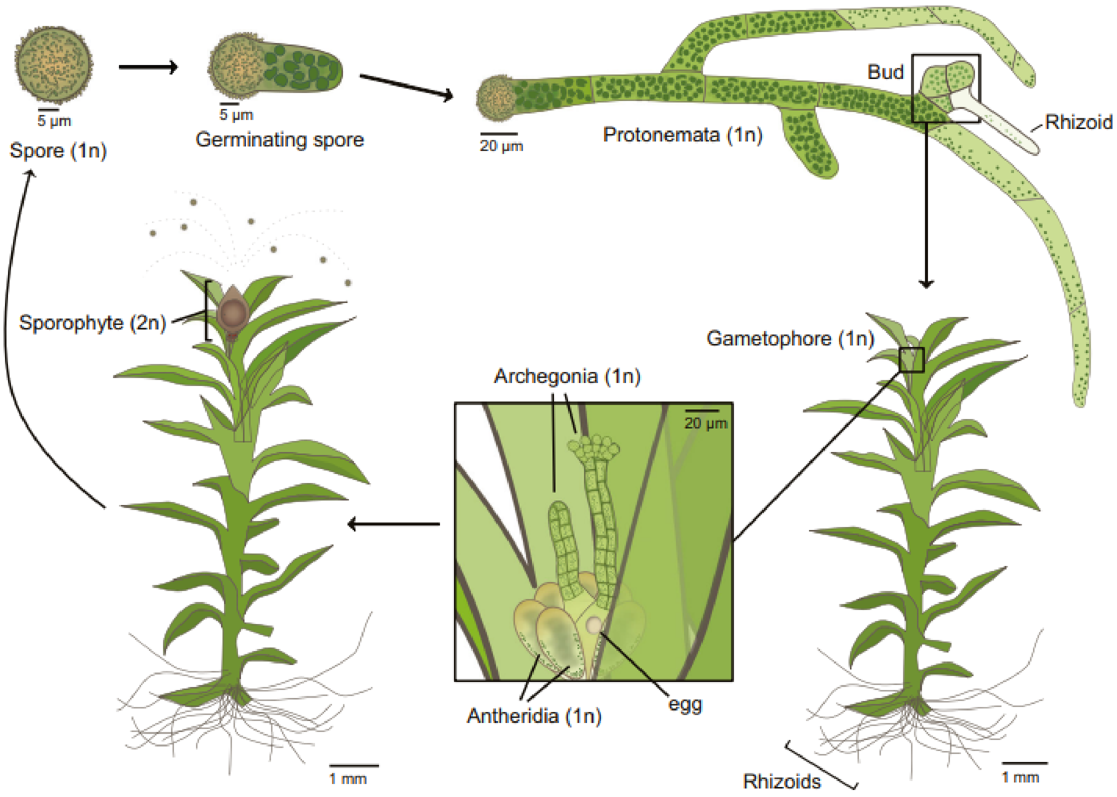


Figure 4. The life cycle of *P. patens* (Wu *et al.,* 2018).

Research Methods

Physcomitrella patens *Growth Conditions*

All samples of *P. patens* will be grown on a heat-sterilized agar BCD medium at 27.5°C and exposed to a fluorescent light with an alternating 12hr light and 12hr dark cycle. To make one liter of BCD medium, 10 mL B stock solution containing 25 grams $MgSO_4 \cdot 7\ H_2O$and 1 L H_2O, 10 mL stock solution C containing 25 grams KH_2PO_4 and 1 L H_2O adjusted to pH 6.5, 10 mL stock solution 0 containing 1.01 grams KNO_3 1.25 grams $FeSO_4 \cdot 7\ H_2O$ and 1 L H_2O, 920 mg di-ammonium (+) tartrate will be combined, diluted to one liter with additional H_2O, then brought to a pH of 6.5 before adding 4 grams Agargel (Sigma Aldridge). To sterilize, the solution will be autoclaved at 121°C at 15 psi for 25 minutes. After sterilization, 1 mL of 1 M $CaCl_2$ will be added and the solution will be poured into Petri dishes and let cool. Propagation of *P. patens* will be achieved by cutting mature samples into 5mm pieces, then placing pieces onto a new BCD plate for growth. Six colonies will be grown on each plate. All tools will be sterilized using 95% EtOH and flame before use.

Jasmonic Acid Treatments

Plants were treated with JA by soaking in solutions containing the desired concentration of jasmonates for either 20 minutes or 1.5 hours. JA was dissolved in 1 ml/gm of acetone, then diluted with water to the desired concentrations: 1mM and 100 uM. Control plants were soaked in a water bath containing acetone of the same concentration used for the treatment groups.

Three plants were soaked in solutions of each concentration and three samples were taken from each plant for analysis.

Mechanical Wounding Treatments

Plants were to be cut in thirds using sterile scissors and then let sit for 5 minutes before homogenization and testing.

Light Intensity Treatments

Three-week-old moss gametophytes were utilized for experimentation (Ponce de Leon *et al.*, 2012). Various light intensity treatments were to be employed: full dark, partial light, and full light. Mosses in the partial and full light treatments were exposed to light for 18 hours and allowed to acclimate to lighting for several days. The light source was fluorescent white bulbs. A full dark environment was achieved by blocking light with aluminum foil. A partial light environment was achieved by using semi-transparent netting. All experiments were conducted at the same time of day at 27.5 degrees C.

Detection of Responses

Detection of JA activity was accomplished by looking at the activity of polyphenol oxidases (PPOs), peroxidases (PODs), lipoxygenases (LOX), and protease inhibitors (PIs). Extraction of enzymes was accomplished by homogenizing leaf samples in 1.25 ml chilled 0.1 M K Phos buffer containing 7% (w:v) polyvinylpolypyrrolidine, then adding 0.4 ml to 10% (v:v) Triton X-100 and vortexing the mixture. The homogenate was centrifuged at 10,000 g for ten minutes and the resulting supernatant was used for spectrophotometric assays of PPO, POD, and LOX activities. In PPO assays, 25 uL of enzyme extract was added to 1 ml of a caffeic acid solution (2.92 mM in pH 8 K Phos buffer). In POD assays, 25 uL of enzyme extract was added to 1 ml of a substrate solution consisting of 2.92 mM guaiacol in pH 8 K Phos buffer with H_2O_2 added as a cofactor. Both PPO and POD assays measured the increase in OD at 490 nm of the mixtures. In LOX assays, the presence of conjugated dienes at 234 nm was measured. The reaction mixture will consist of 15 uL of enzyme extract added to 2.9 ml of 0.4 mM linoleic acid dispersed in a 0.1 M K Phos buffer (pH 7) containing Tween-20 (0.1%). Changes in absorbance will be monitored for at least 5 min (Thaler *et al.,* 1996).

To test for the presence of PIs, an assay was performed that detects the inhibition of the digestion of the artificial protease substrate benzoyl tyrosine ethyl ester (BTEE) by commercial proteases. Mosses were ground in a 50 mM Tris HCl buffer (pH 7.8, 3 ml/gm leaf tissue) containing 7% polyvinylpolypyrrolidine, 1.67 mM phenylthiourea, 0.3 M KCl, and 0.4 mM ascorbic acid to extract proteinase inhibitors. For assays, the extract was centrifuged at 13,000 g for ten min and the supernatant was used as the source of PIs. In a small cuvette, 25 uL of the PI extract was added to 25 uL of 0.001 N HCl containing 0.001 mg of chymotrypsin, and the mixture was allowed to incubate for ten min. After incubation, 2.9 ml of 0.5 mM BTEE was added to the cuvette and the increase in absorbance at 256 nm was measured. A control of chymotrypsin and BTEE without moss extracts acted as a control. PI activities of samples were expressed as % chymotrypsin activity relative to this control (Thaler *et al.,* 1996).

Results

Use of Exogenous JA to Trigger JA Pathway

Before further experiments could be performed, the ability of *P. patens* to produce a defense response using the JA pathway had to be determined. Ideally, exposure to the hormone would trigger a robust production of PPOs, PODs, and LOXs. Plants were exposed to varying concentrations of JA for either 20 minutes or 1.5 hours and the plant was homogenized for enzyme extraction. Once extracted, the enzyme activity was measured using spectrophotometry. Exposure of *P. patens* to exogenous JA appeared to increase the activity of PPOs (Figure 5 & 6) but did not affect the activity of LOXs. Despite repeated efforts, the exposure of *P. patens* to exogenous JA did not result in the production of significant amounts of the measured enzymes at the concentrations used in this experiment. Due to accidents while moving labs, a significant amount of data was lost, including all experiments examining the result of exogenous JA on PPO and LOX activity. What data is present likely does not accurately depict the relationships between JA exposure and enzyme activity (Figure 5 & 6).

Use of Other Chemical Elicitors to Trigger Defense Response

Due to the lack of response seen in response to exposure to JA, other chemical elicitors were used as well, including salicylic acid and chitosan. *P. patens* samples were exposed to SA and chitosan and enzymes were extracted in similar methods to the JA experiments. A chitosan concentration of 1mg/ml and an SA concentration of 1 mM was used. Similar to the JA experiments, no significant production of enzymes was found with the concentrations tested. Remaining data suggest that exogenous JA results in greater POD activity than exogenous chitosan (Figure 6)**.** Data from the experiments using SA were lost along with experiments examining the effect of all elicitors on PPOs and LOXs.

Mechanical Wounding to Trigger Defense Response

This experiment was not performed due to the lack of activity in the JA pathway determined by the previous experiments.

Light Intensity Treatments

This experiment was not performed due to the lack of activity in the JA pathway determined by the previous experiments.

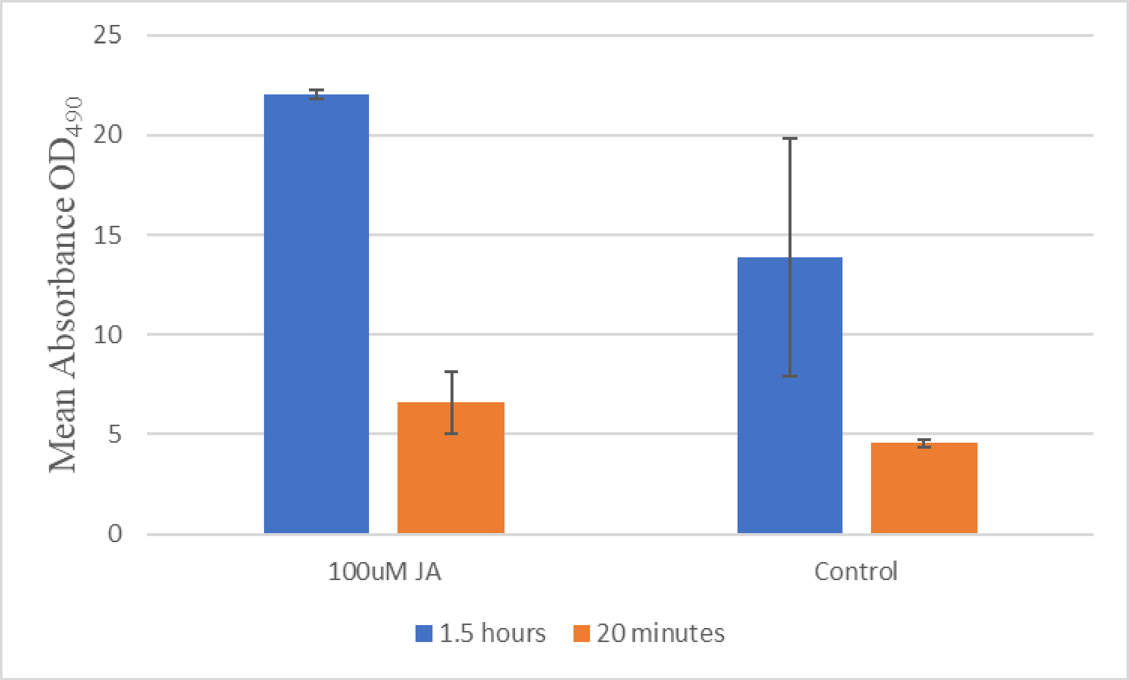


Figure 5. Comparison of peroxidase activity in *P. patens* after either 20 minutes or 1.5 hours of exposure to 100uM JA. The experiment was performed with 15uL of enzyme extract and 200uL guaiacol. (Error bars represent ± SE, n = 3).

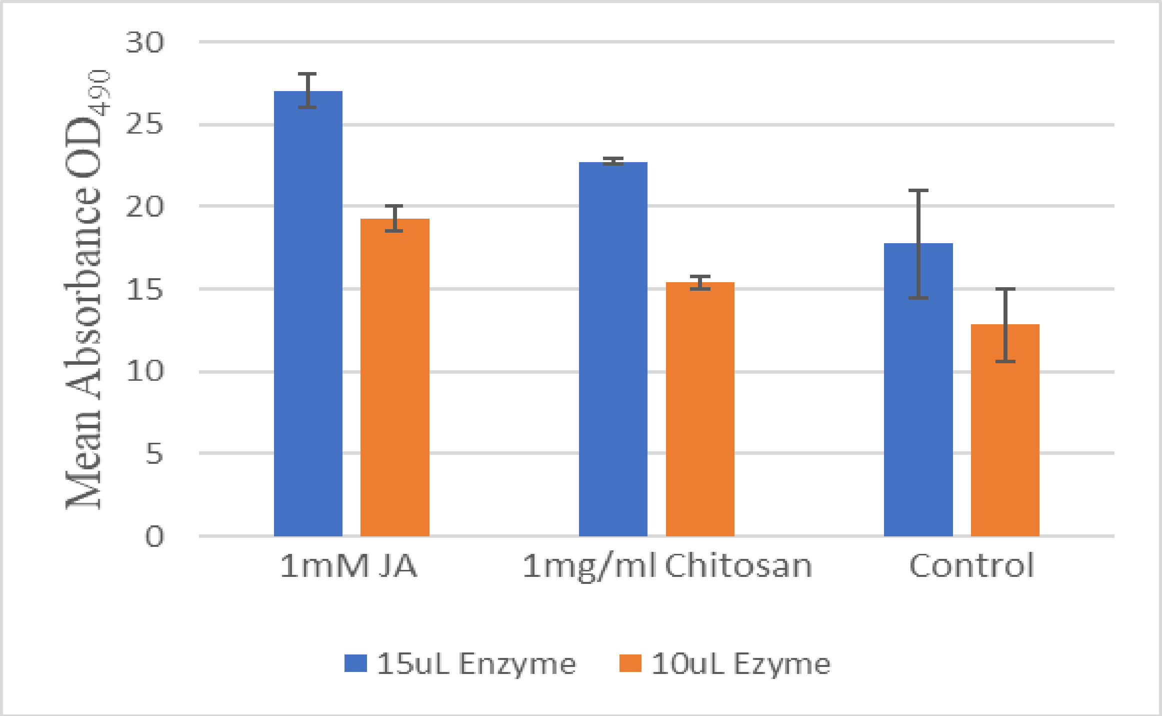


Figure 6. Comparison of peroxidase activity in *P. patens* after 1.5 hours of exposure to 1mM JA, 1mg/ml Chitosan, or water. Different colored bars represent experiments performed at either 10uL enzyme extract and 200uL guaiacol or 15uL of enzyme extract and 200uL guaiacol. (Error bars represent ± SE, n = 3).

Discussion

As a result of plants being the target of a multitude of attacks from herbivores and pathogens alike, they have developed various defense mechanisms to protect themselves. These defenses range from external thorns and trichomes to internal chemicals. The jasmonic acid pathway is a common inducible defense that has been well characterized in many vascular plants. These vascular plants, such as most agricultural plants, have been heavily studied due to their economic importance. Similarly, much research exists regarding the impact of light on this defense pathway in vascular plants (Kazan & Manners, 2011).

Chemical Elicitors

The presence of this pathway in lower plants, such as bryophytes, is less studied and current studies often present conflicting results. Several studies support the presence of JA in *P. patens* and other bryophytes while others have disconfirmed this finding (Stumpe *et al.,* 2010; Bandara *et al.,* 2009). The presence of OPDA, the JA precursor, has been noted in *P. patens* as well. (Stumpe *et al.,* 2010). As the current study does not directly measure the presence or absence of these compounds in *P. patens*, it cannot support either conclusion. This study does provide evidence that JA likely does not play the same role in *P. patens* as it does in other bryophytes or vascular plants, though the low sample size due to lost data may prevent any definitive conclusions. It is possible that within *P. patens*, JA impacts growth and development, but does not play a role in the inducible defense of the moss. Another possibility is that it does still play a role in defense, but that role involves the production of proteins not examined in this research.

Alternatives to the Jasmonic Acid Pathway

Due to their small size and simple structure relative to vascular plants, bryophytes often lack the ability to create the complex physical structures that make up the robust constitutive defenses that many vascular plants possess. However, their small size relative to the vascular plants around them also protects them from the view of herbivores, leading to the bryophytes being overall less consumed than other kinds of plants (Feeny, 1976; Glime, 2006; Markham *et al.,* 2006). The unlikeliness of an attack from an herbivore leads to the investment of chemical defenses over the more expensive physical structures (Chen *et al.,* 2021).

The secondary metabolites that bryophytes depend on make up both constitutive and inducible defenses. The most common metabolite used are terpenoids, a class of chemicals derived from the 5-carbon molecule, isoprene (Peters *et al.,* 2019). Terpenoids function very similarly to other secondary metabolites, acting as general antifeedants and attracting the predators of the herbivores (Kappers *et al.,* 2005). Other metabolites are also common within the tissues of bryophytes. In a study of metabolites from nine moss species, Peters *et al.* (2019), determined that the most abundant metabolites were those that were related to species-specific responses. They also determined that metabolites in these groups increased in concentration during growing seasons, suggesting a period of greater interaction with other organisms during these times.

Light Intensity

Very little, if any, research exists examining the impact of light intensity on the chemical defenses of bryophytes in response to herbivory. Further investigation into this topic should be performed. To gain insight into the impact of light on the defense systems of bryophytes, it may be useful to examine the same in vascular plants, where research is plentiful. Research into the importance of light on the activity of plants falls into two main categories: studies on resource allocation and studies on light signaling.

The core essence of plant growth and survival is a balance of resource allocation. For a plant to be successful, it must have an optimal distribution of nutrients. Competition and stress, both abiotic and biotic, are factors that a plant must address. A plant has to grow fast enough to outcompete its neighbors and receive enough sunlight and nutrients, and it also has to have enough resources to defend itself from a variety of herbivores and pathogens. In this case, light is immensely important (Ballaré, 2014). It can provide the plant with the energy, through photosynthesis, to perform both of these actions. In these cases, the obvious conclusion is that the greater the amount of sunlight a plant receives, the better off it is. The plant would be able to perform the maximum amount of photosynthesis to create as much sugar for growth and defense as possible. Roberts & Paul (2006) concluded that herbivores feeding on plants in the shade were more successful than those feeding on plants that received full sunlight, suggesting a stronger defense from plants with more access to light. However, some plants may also have to worry about too much sunlight, as too much can lead to excessive transpiration, or loss of water through the stomata, the small openings on the surface of leaves that allow for gas exchange (Pallardy, 2008). Once again, the plant must balance its resources.

Light also acts as a source of information regarding the plant's environment. For example, light signals can provide the plant with information regarding the activity and density of nearby plants (Ballaré, 2014). Phytochrome B, the main photoreceptor that allows plants to detect the presence of nearby plants, uses the ratio of red to far-red light (R:FR) (Ballaré, 1999). In the absence of competitors and shade, the plant receives blue, red (R), and far-red (FR)light from the sun. While blue and red are absorbed, FR is transmitted or reflected. In this scenario, the R:FR is about 1.2. (Smith, 1995). In these conditions, phytochrome B is activated and found in the nucleus preventing the accumulation of growth factors (Leivar & Quail, 2011). When the plant is in shade, with a low R:FR, phytochrome B is inactivated (Smith, 1995). This leads to a signal cascade which eventually leads to the production of auxin, and the growth of the plant in an attempt to obtain more light and outcompete its neighbors (Li *et al.,* 2012).

Similarly, the exposure of plants to a low R:FR, can decrease the plant's defense system. Plants exposed to low R:FR and plants with a mutated phytochrome B produced an attenuated defense response when tested (Cerrudo *et al.,* 2012; Moreno *et al.,* 2009). This is thought to be an interaction with the inactivated phytochrome B and JA. Studies have shown that mutating phytochrome B leads to a decrease in the expression of the JA pathway, significantly decreasing the accumulation of defensive metabolites (Ballaré *et al.,* 2012). Another study from Kegge *et al.* (2013) determined that low R:FR as a result of shading led to a decrease in both constitutive and JA-related compounds in *Arabidopsis thaliana*, a common model organism for flowering plants.

This data suggests that a vascular plant's defense system is susceptible to decreases in light intensity due in part to their use of the JA pathway. As this experiment and other studies suggest that JA does not play a role in bryophyte defense it may be possible that bryophytes are less impacted by changes in light exposure.

Conclusions and Future Directions

By exposing the bryophyte, *Physcomitrella patens*, to exogenous jasmonic acid, the production of peroxidases, polyphenol oxidases, lipoxygenases, and protease inhibitors was studied. While few conclusions can be drawn from the experiments performed, due to loss of data and subsequent small sample size, the data presented in this study do still support previous studies investigating the absence of jasmonic acid-related defense signaling in bryophytes. The results from this study do not support the hypothesis that bryophytes can detect and respond to their environment using the jasmonic acid pathway. It may be useful in the future to retry the experiment to obtain more data. Additionally, future research should examine the response of other defense-related compounds after exposure to exogenous jasmonic acid. In regard to light exposure and its impact on the defense systems of bryophytes, little research currently exists on the topic. This area of study may benefit from the study of the impact of light intensity on the ability of bryophytes to produce well-established defense responses, such as the production of terpenoids.

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