The Role of Calcium in Pathogen Defense Responses in the Moss Mnium cuspidatum

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The Role of Calcium in Pathogen Defense Response in the Moss *Mnium cuspidatum*

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In Partial Fulfillment of Requirement for
Graduation Honors

Morgan Claire Blake
April 29, 2016
Dedication

This thesis is dedicated to my family and my friends, especially to my parents Kenny and Maggie Blake. I would also like to dedicate this thesis to all those who have continued to encourage and support me in the past year.
Acknowledgements

I would like to extend my gratitude to Dr. Philip Villani for his guidance and patience, the Hauck Lab for company, knowledge, and materials, the Biology Department, and the Dr. Lantzer and the rest of the Honors Department for their unwavering support.
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Abstract

When exposed to stress, whether that be by pathogen, thermal or weather changes, or other components of the environment, plants employ a variety of defense mechanisms depending on the severity of the stress. When attacked by a pathogen, the moss *Mnium cuspidatum* utilizes an innate, basal response followed by hypersensitive response (HR) and systemic acquired resistance (SAR). Calcium is known to be involved in signaling cascades that mediate defense responses in other organisms. In the present study, lanthanum nitrate (LaNO₃), methoxyerapamil (D600), and ethylene glycol tetraacetic acid (EGTA) were used to inhibit the presence of calcium in *M. cuspidatum* in the presence of the fungal pathogen *Pythium irregulare* in efforts to discover the relative involvement of calcium in innate immunity and HR. Results suggest that calcium in fact mediates signal transduction pathways utilized in such defense mechanisms. When grown on BCD medium containing these inhibitors and then subsequently infected with *P. irregulare*, the moss presented cleared-out leaves, browning stems, congregating chloroplasts, reactive oxygen species (ROS) production, and reinforcement of cell walls with the production and linking of polysaccharides—all events which are characteristic of innate basal response and HR.
**Abbreviations**

MAMP: Microbe-Associated Molecular Pattern

HR: Hypersensitive Response

PCD: Programmed Cell Death

ROS: Reactive Oxygen Species

SAR: Systematic Acquired Resistance

EtOH: Ethanol

PDA: Potato Dextrose Agar

EGTA: Ethylene Glycol Tetraacetic Acid

DAB: 3-3’-diaminobenzidine

CTC: Chlortetracycline

PCR: Polymerase Chain Reaction
Introduction

Defense Mechanisms

Every life form on Earth has the potential to get sick. People get sick, our pets get sick, even plants get sick. Thus, it is important to understand exactly what it is that makes organisms sick and what biological defense mechanisms are utilized to combat illness. We know that microscopic pathogens are the cause of many diseases, but it is still unclear as to what mechanisms are utilized by some organisms in pathogen defense.

Throughout the course of evolution, organisms have developed intricate defense mechanisms to combat infection. Recent studies indicate that some of these mechanisms, are conserved across the plant and animal kingdoms (Ausubel, 2005). For example, both plants and animals respond to pathogen-associated molecular patterns (PAMPs) / microbe-associated molecular patterns (MAMPs) through pathogen-recognition receptors. In vertebrate animals, this innate immunity is the first line of defense and also the precursor to production and activation of certain immune components such as T cells (Ausubel, 2005).

The vast majority of pathogenic species are microorganisms. Whether they be bacteria, viruses, fungi, or parasites, organisms need successful and sufficient defense mechanisms against these species to thrive. It is important to note that not all microorganisms are pathogens, but those that are generally have wide spread effects of varying severities on their hosts. For example, fungal pathogens such as dermatophytes cause the well-known superficial infections of the skin such as athlete’s foot, ringworm, and nail infections. Other fungal species such as those belonging Cryptococcus, Candida,
and *Aspergillus* cause more invasive infections that may even lead to death (Brown et al., 2012).

The World Health Organization estimated that 15 million people died from infectious disease in 2010, a decrease from 16 million in 1990. It is estimated that this number will fall to 13 million by 2050 (Dye, 2014). Plants, too are susceptible to pathogenic infection, specifically in agriculture. It is estimated that almost 40% of worldwide crops are lost to pathogenic disease each year (Strange, 2012; Pinstrup-Andersen, 2001). Thus, it is important to continue study of pathogenic infections in lower organisms in order to further develop our knowledge base of conserved defense mechanisms.

**Plant-Pathogen Interaction**

In plants, the first line of defense is structural. All plants contain a waxy outer cuticle that covers leaves and bark and deters pathogens and other predators. Some have additional structures such as hairs, thorns, and spines. Internally, plant cells and their primary and secondary cell walls provide extra lines of structural defense against microorganisms. However, as pathogens will inevitably penetrate these barriers, additional defense mechanisms are necessary.

Plants have the ability to recognize generic microbe-associated molecular patterns (MAMPs) such as certain proteins and cell wall components like chitin (Ausubel, 2005; Freeman and Beatie, 2008; Iriti and Faoro, 2009). Scientists have developed derivatives of MAMPs such as chitosan, a deacetylated chitin derivative, to induce defense responses such as innate immunity (Iriti and Faoro, 2009). This response is characterized by an
attempt by the plant to prevent early colonization of the invading pathogen (Grennan, 2006). Such response usually results in fortification of plant cells against future similar attacks. Should this initial basal immune response be overcome, plants may employ a hypersensitive response (HR).

HR is an intrinsically-programmed process that is characterized by rapid, intentional programmed cell death (PCD; apoptosis) of healthy cells in the regions immediately surrounding the site of infection (Morel and Dangl, 1997). It is elicited when gene products in the plant cell identify certain disease-causing effector molecules. This effectively limits the invading pathogen’s access to the water and nutrients it needs to spread to additional plant regions (Freeman and Beatie, 2008). With the naked eye, one may be able to observe HR in the form of a single dead leaf on an otherwise healthy plant. Within the cells of this leaf, one could observe cell wall thickening, defense chemical production, chloroplast congregation, and PCD. Generally, these events of HR are successful in preventing the spread of disease as they restrict the pathogen’s access to nutrient-rich tissues.

In conjunction with the early defense mechanisms of basal response and HR, plants often respond with rapid production of reactive oxygen species (ROS), a so-called oxidative burst that may also be triggered by the presence of MAMPs. This response has two main functions: 1) strengthen and fortify cell walls by linking existing structural components such as glycoproteins and lignin, and 2) activate signaling cascades to turn on defense gene expression (figure 1) (Torres et al, 2006).
An additional defense mechanism utilized by plants is systemic acquired resistance (SAR). SAR involves a signal transduction pathway that becomes activated in response to an initial infection, which then enables a plant to defend itself against future pathogen infections (Ryals et al, 1996).

**Role of Calcium in Defense Mechanisms**

Previous studies in plant pathology suggest that calcium is also important in signal transduction pathways mediating immune and defense responses in a variety of organisms across phylogeny. In *Drosophila melanogaster* and *Caenorhabditis elegans*, both model organisms, calcium has been shown to be the initial trigger in immune

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**figure 1. Reactive oxygen species (ROS) production and function in response to pathogens.** Plants recognize pathogens by their microbe-associated molecular patterns (MAMPs) such as certain cell wall components. Plants may also recognize human-synthesized pathogen elicitors such as chitosan and subsequently counter with ROS and related defense mechanisms (Torres et al, 2006).
responses regarding wound repair (Razzell et al, 2013). Further, Levine (1996) concluded that calcium aids in defense by activating programmed cell death resulting in DNA fragmentation, cell shrinkage, and nuclear condensation in soybeans, typical of HR. However, there is no current literature regarding the role of calcium in defense mechanisms utilized by mosses.

**Calcium Inhibitors**

In order to study the necessity of calcium in any system, one should completely block its presence. In this plant-pathogen interaction system, calcium was blocked with three different inhibitors: lanthanum nitrate (LaNO₃), methoxyverapamil (D600), and ethylene glycol teraacetic acid (EGTA). Each inhibitor acts to block prevent the plants’ use of calcium by means of a different mechanism.

LaNO₃ is a lanthanum derivative and has been suggested to inhibit calcium-permeable, but non-specific, cation channels that are activated in response to alterations in calcium concentration (Demidchik et al, 2002). D600 is a derivative of verapamil and acts as a calcium ion channel antagonist (Singh et al, 2010). That is, it acts to inhibit calcium signaling by binding to intracellular calcium ion channels and preventing the secondary signaling of calcium. Finally, EGTA is a well-known calcium chelator and acts to inhibit calcium by binding surrounding calcium before being absorbed by the system. Specific to this research, EGTA binds calcium in the medium before being absorbed by moss.
Vascular Plants

Vascular plants may also be referred to as higher plants as they are the most recent evolutionary division of plants. They are characterized by vascular tissue systems that include xylem to transport water to photosynthetic parts of the plants as well as phloem to transport sugars produced by photosynthesis (Moore et al, 1998).

Bryophytes

In contrast to vascular plants, bryophytes, consisting of mosses, liverworts, and hornworts, are non-vascular and do not contain specialized vascular tissues. Therefore, they grow close to the ground where water and nutrients are easily accessible. Root-like rhizoids aid in anchoring the plants to their surroundings. Bryophytes’ single sheets of cells aid in ease of nutrient absorption and photosynthesis. These are generally referred to as “lower plants” as they evolved before any other group of plants.

Bryophytes also differ from higher-order vascular plants with regard to their lifecycle. Mosses, for example, begin as a single haploid spore that produces a filamentous chain of cells that grows to form a dioecious gametophore. When male and female gametophores meet, sperm and egg combine to form a diploid zygote. This zygote will ultimately grow into a stalk from which haploid spores are released and the cycle repeats itself (figure 2) (Campbell et al, 2008).
figure 2. The lifecycle of moss. The cycle begins with haploid spores that transition into male and female gametophores. The female gametophore is fertilized by the male gametophore and ultimately the cycle ends with diploid gametophytes (Campbell et al, 2008).
Mosses in particular play a significant role in our ecological and evolutionary understanding of life on Earth as they are the most ancient form of terrestrial plant life. Mosses are known to be the simplest land plants and are therefore frequently used as model systems (Cove, Knight, and Lamparter, 1997). Previous studies have found that mosses induce conserved defense mechanisms in response to pathogens, though this has not been specified in *M. cuspidatum* in particular (Oliver et al, 2009).

Overall, further investigation into the defense mechanisms in lower eukaryotic organisms such as moss could lead us to a greater understanding of defense mechanisms utilized by higher plants and possibly other eukaryotes such as humans (Morel and Dangl, 1997).

*Mnium cuspidatum*

Of the mosses used in research, *M. cuspidatum* (sometimes also referred to as *Plaginmnium cuspidatum*) is perhaps the least studied and characterized. In fact, no previous research exists regarding the aforementioned defense responses in the moss. However, it serves as an ideal organism in this study due to the nature of its growth. *M. cuspidatum* is characterized by long stems and individual, spaced single-cell-layer leaves. The long stems of *M. cuspidatum* allow for easy application of pathogen species and chemical inhibitors and subsequent observation of local defense responses such as HR and ROS as well as gradient and delayed defense responses such as SAR. Observation of HR and ROS is also made easier as leaves are separated and easily distinguishable on the stems.
Pythium irregulare

The pathogen used in this study is *P. irregulare*, a soilborne fungus. The fungus is reported to thrive on all continents except Antarctica, and tends to be found on many agrarian plant species in the United States (Katawczik, 2008). Upon infection with *P. irregulare*, plants both in the field and in greenhouse have been shown to develop a general plant disease known as blight as well as root rot. Further, Oliver et al (2009) state that mosses employ conserved defense mechanisms upon infection with *P. irregulare*, though not specified in *M. cuspidatum*. *P. irregulare* can be identified under compound light microscopy by its characteristic thin, filamentous hyphae.

Thesis Research and Hypothesis

Calcium is an important signaling molecule in higher-order, vascular plants. Levine et al (1996) concluded that calcium aids in defense by activating programmed cell death resulting in DNA fragmentation, cell shrinkage, and nuclear condensation in soybeans, typical of HR. However, there is no literature regarding the role of calcium in defense mechanisms utilized by mosses.

Given that many plant-pathogen interactions and defense mechanisms are conserved among the kingdom, I expect that calcium plays a similar role in *M. cuspidatum*. Specifically, I expect that the removal of calcium from the moss should inhibit its ability to ward off a pathogen. If calcium is in fact involved in the HR of *M. cuspidatum*, I would expect to see increase production of reactive oxygen
species such as H$_2$O$_2$, phenolics, and cell wall polysaccharides in response to infection. Further, chloroplast congregation should also be observed.

Understanding these topics will allow for further understanding of the defense mechanisms utilized by *M. cuspidatum*, which may ultimately aid in our understanding of defense mechanisms utilized by all eukaryotes. Furthermore, not only will answering such questions further our understanding of plant evolution, but may lead to implications that could benefit agronomic plants as well.
Materials and Methods

Plant and Pathogen Growth

*M. cuspidatum* was grown in Dr. Philip Villani’s laboratory at 26°C under a photoperiod of 16 hours under fluorescent lights on sterilized BCD medium. Stock BCD medium was prepared in 500 mL increments with 5 mL stock solution B made from 25 g MgSO₄·7H₂O filled to 1 L with distilled H₂O, 5 mL stock solution C made from 25 g KH₂PO₄ and 500 mL distilled H₂O adjusted to pH 6.5 with 4M KOH, 5 mL stock solution D made from 101 g KNO₃ and 1.25 g FeSO₄·7H₂O filled to 1 L with distilled H₂O, 0.5 mL trace mineral solution made from 614 mg H₃BO₃, 110 mg AlK(SO₄)₂·12H₂O, 55 mg CuSO₄·5H₂O, 28 mg KBr, 28 LiCl, 25 mg Na₂MoO₄·2H₂O, 389 mg MnCl₂·4H₂O, 55 mg CoCl₂·6H₂O, 55 mg ZnSO₄·7H₂O, 28 mg KI, 28 mg SnCl₂·2H₂O, and 59 mg NiCl₂·6H₂O filled with distilled H₂O to 1 L, and 460 mg diammonium tartrate. The solution was then brought to a volume of 500 mL by distilled H₂O and adjusted to pH 6.5 with 4M KOH before the addition of 2 g phytagar (Cove, 2004). The solution was then microwaved for 4 minutes and then swirled to dissolve agar before autoclaving for 60 minutes. Following autoclave sterilization, 0.5 mL CaCl₂ was added and then the agar solution was poured into petri dishes and let sit to solidify before use.

*M. cuspidatum* was maintained and propagated using utensils flamed sterilized with 70% EtOH each time before use. Fully grown moss was sectioned into approximately 1 cm pieces and removed from stock plates and transferred to new petri dishes. Approximately 5 pieces were on each dish. Dishes were sealed with Parafilm M to ensure moisture retention.
*P. irregulare* was used to elicit fungal infections in *M. cuspidatum*. *P. irregulare* was obtained from sterile samples maintained in Dr. Villani’s laboratory. The fungus grew on the standard fungal medium Potato Dextrose Agar (PDA) at 1/8 strength so that the fungus maintained pathogenicity due to unsatisfactory amounts of nutrients. PDA solution at 1/8 strength was prepared from 2.4 g stock PDA containing 15 g/L agar, 20 g/L dextrose, and 4 g/L potato extract and 6.5 g BD Difco agar filled to 500 mL with distilled H₂O. The agar solution was then microwaved for 6 minutes to boil and subsequently separated into 5-100 mL flasks and autoclaved 60 minutes. Stock *P. irregulare* samples were maintained in shade at 24°C.

**Preparation of Calcium Inhibitors**

Three different calcium inhibitors were used in investigating the role of calcium in moss and fungus growth: lanthanum nitrate (La(NO₃)₃), methoxyverapamil (D600), and ethylene glycol tetraacetic acid (EGTA). A 1 mM stock solution of La(NO₃)₃ was prepared from 32.5 mg La(NO₃)₃ in 100 mL distilled H₂O. A 50 mM stock solution of D600 was prepared from 801 mg D600 in 100 mL distilled H₂O. A 500 mM stock solution of EGTA was prepared with 4.75 g EGTA in 25 mL H₂O then followed by an addition of 1.66 g NaOH before bringing the solution to pH 8 with 1 M HCl. An inhibitor cocktail was also prepared in 100 mL distilled H₂O using the above amounts of each inhibitor. All inhibitors were obtained from Sigma Aldrich.
Role of Calcium in *M. cuspidatum* growth

To investigate whether or not calcium mediates the growth of *M. cuspidatum*, LaNO$_3$, D600, and EGTA were added directly to hot BCD medium. 500 mL BCD was prepared as described, but evenly split into 100 mL portions directly before autoclaving. Immediately after autoclave sterilization, 5-100 mL BCD solutions were prepared as follows: 1) 100 mL BCD + 400 µL CaCl$_2$ + 100 µL LaNO$_3$, 2) 100 mL BCD + 400 µL CaCl$_2$ + 10 µL D600, 3) 100 mL BCD + 400 µL CaCl$_2$ + 2 mL EGTA, 4) 100 mL BCD + 400 µL CaCl$_2$ + 100 µL LaNO$_3$ + 10 µL D600 + 2 mL EGTA, 5) 100 mL BCD + 400 µL CaCl$_2$. Five new dishes were poured for each of the aforementioned agar solutions and let sit to solidify before use.

*M. cuspidatum* was transferred to these plates using utensils flamed-sterilized with 70% EtOH each time before use. Approximately 1 cm sections were cut from the stem termini and placed on a dish, and the dish was sealed with Parafilm M. The meristem location was noted on the dish with permanent marker. Each dish had only 1 specimen. Specimen growth was marked and measured at 2 weeks and 4 weeks. Measurements were then averaged per treatment.

Role of Calcium in *P. irregulare* growth

To investigate whether or not calcium mediates the growth of *P. irregulare*, LaNO$_3$, D600, and EGTA were added directly to hot PDA medium. 500 mL PDA was prepared as described, but split into 100 mL portions directly before autoclaving. The same method as described in the above section was followed to add inhibitors to PDA medium immediately after autoclave sterilization, excepting the addition of CaCl$_2$. Three
plates per treatment were poured and let sit to solidify before use. A single *P. irregulare* plug from stock plates was then added to each treated plate. Growth was marked and measured each day for 3 days. Measurements were then averaged per treatment.

**Inoculation of *M. cuspidatum***

*M. cuspidatum* was transferred from dishes of stock specimens to 3 new BCD control plates. Each plate contained approximately 3-1 cm pieces of stem termini, and each sample was then inoculated with a *P. irregulare* via a small agar plug placed directly onto the cut end of the stem. Specimens were observed within 24 hours with the staining techniques described in the following section.

**Interaction between *M. cuspidatum* and *P. irregulare***

To investigate whether or not *P. irregulare* infected the *M. cuspidatum* samples, the described specimens were stained and observed within 24 hours of inoculation. Stains included 0.5% toludine blue, lacto phenol blue, 0.01% safranin, and DAB. For toludine blue staining: specimens were extracted from the BCD plates, stained with 0.5% toludine blue for 5 minutes, rinsed with distilled H$_2$O until all excess stain washed away, and then placed on microscope slide with distilled water. For lacto phenol blue staining: specimens were extracted from BCD plates, placed directly on microscope with water and stained with lacto phenol blue. For safranin staining: specimens were extracted from BCD plates, stained with 0.01% safranin for 4 minutes, rinsed with distilled H$_2$O until all excess stain removed, then placed on a microscope slide with water. For DAB Staining: specimens
were extracted, bleached in ethanol for 24 hours, and then placed directly on slides with DAB. All observations were made using a standard compound light microscope.

**Role of Calcium in *M. cuspidatum* Hypersensitive Response**

*M. cuspidatum* was also transferred to plates with each of the 4 different BCD + inhibitor treatments. Three plates per treatment were prepared. Each new plate had 3-1 cm terminal pieces. The samples were then inoculated with *P. irregulare* plugs and exposed for 12-24 hours and then observed with dyes and microscopy as described above. In some trials, *M. cuspidatum* was exposed to 1 mg/mL chitosan for 24 hours to elicit defense responses, though defense molecule production was not evident with microscopy (not shown).

**Determination of Apoptosis**

To observe the chemical presence of and role of calcium in HR on the cellular level as well as the presence of apoptosis, various microscopy and staining techniques were used. Basic compound light microscopy was used to observe congregation of chloroplasts. DAB stains were used to determine presence of ROS in plant tissue while toluidine blue staining was used to determine the presence of cell wall polysaccharide production and phenolics. Lacto phenol blue and safranin staining did not produce observable results (not shown). Exact staining techniques for DAB and toluidine blue are described above. Finally, autofluorescence of chloroplasts and phenolics was observed with a Leica DMLB in conjunction with an ExiQ imaging camera and Metamorph v7.78
software. Slides for all microscopy techniques were prepared by placing a >1 cm specimen on slide with water and cover slip.
Results

Role of Calcium in *M. cuspidatum* growth

*M. cuspidatum* was grown on BCD medium under the following treatments: 1) BCD, 2) BCD + 1 mM LaNO$_3$, 3) BCD + 50 mM D600, 4) BCD + 500 mM EGTA, 5) BCD + 1 mM LaNO$_3$ + 50 mM D600 + 500 mM EGTA. The amounts of inhibitors used per 100 mL BCD stock are aforementioned. The final concentrations of the inhibitors used are 1mM LaNO$_3$, 50mM D600, and 5 mM EGTA. Moss was grown under each treatment for 2 weeks before measuring growth of the meristem. No inhibitor treatment under the aforementioned concentrations resulted in statistically significant growth (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>BCD</th>
<th>BCD + LaNO$_3$</th>
<th>BCD + D600</th>
<th>BCD + EGTA</th>
<th>BCD + all 3 inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 2 week growth (mm)</td>
<td>5.27 ± 2.78</td>
<td>5.00 ± 0.12</td>
<td>4.77 ± 1.98</td>
<td>1.33 ± 1.40</td>
<td>2.95 ± 2.13</td>
</tr>
</tbody>
</table>

Table 1. *Mnium cuspidatum* growth with treatment of calcium inhibitors. The moss was grown on BCD medium with different inhibitor treatments to assess the inhibitors’ effects on the growth of the moss. Three replicates of each treatment were performed: 1) control: BCD medium as described above, 2) BCD + 1 mM LaNO$_3$, 3) BCD + 50 mM D600, 4) BCD + 5 mM EGTA, 5) BCD + 1 mM LaNO$_3$ + 50 mM D600 + 5 mM EGTA. Growth was measured from the original placement of the meristem. A student t-test revealed that none of the treatments resulted in statistically significant differences in growth as compared to the BCD control (all p > 0.05).

Role of Calcium in *P. irregulare* growth

*P. irregulare* was grown on 1/8$^{th}$ PDA medium under the following treatments: 1) 1/8$^{th}$ PDA, 2) 1/8$^{th}$ PDA + 1 mM LaNO$_3$, 2) 1/8$^{th}$ PDA + 50 mM D600, 4) 1/8$^{th}$ PDA + 500 mM EGTA, 5) 1/8$^{th}$ PDA + 1 mM LaNO$_3$ + 50 mM D600 + 500 mM EGTA. Per 100
mL stock of 1/8<sup>th</sup> PDA, the same amounts of each inhibitor were used as in the BCD treatments and are aforementioned.

Three plates of fungus were grown under each treatment for 3 days total. Growth was measured every 24 hours and then averaged; no treatment resulted in statistically significant differences in overall 3-day growth (Table 2). *P. irregulare* treated with 500 mM EGTA and with the 3-inhibitor cocktail completely ceased to grow. Thus, the concentration was decreased to 5 mM for plant-pathogen interaction experiments to ensure the growth of *P. irregulare* on all treatments (data not shown).

<table>
<thead>
<tr>
<th>Mean 3 day growth (mm)</th>
<th>PDA</th>
<th>PDA + LaNO&lt;sub&gt;3&lt;/sub&gt;</th>
<th>PDA + D600</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>68.33 ± 2.52</td>
<td>67.67 ± 3.06</td>
<td>66.33 ± 0.58</td>
</tr>
</tbody>
</table>

**Table 2.** *Pythium irregulare* growth with treatment of various calcium inhibitors. The fungus was grown on PDA medium with different inhibitor treatments to assess the inhibitors’ effects on the growth of the fungus. Three replicates of each treatment were performed: 1) control: PDA medium as described above, 2) PDA + 1 mM LaNO<sub>3</sub>, 3) PDA + 50 mM D600, 4) PDA + 5 mM EGTA, 5) PDA + 1 mM LaNO<sub>3</sub> + 50 mM D600 + 5 mM EGTA. Data for the EGTA and 3-inhibitor treatment is not shown. Every 24 hours for the next 72 hours, radial growth was measured from the growing front. A student t-test revealed that none of the treatments resulted in statistically significant difference in overall 3-day growth of the *P. irregulare* as compared to the PDA control (all p > 0.05).

**Role of Calcium in *M. cuspidatum* Hypersensitive Response**

To investigate the role of calcium in *M. cuspidatum* HR, moss was plated on the same treatments outlined above, inoculated with *P. irregulare*, and grown for 24 hours before staining.

Toludine blue staining was used to indicate the location of HR and the presence of fortifying cell wall polysaccharides and phenolics in individual moss leaves. Purple staining represents modifications to cell wall polysaccharides and blue staining represents...
production of phenolics in response to infection. All moss treated with inhibitors and exposed to *P. irregulare* appeared to show increased infection as compared to the BCD control (Figure 3).
Figure 3. Toluidine blue staining of infected *M. cuspidatum* treated with calcium inhibitors. *M. cuspidatum* was exposed to *P. irregulare* for 24 hrs then stained with toluidine blue to indicate a hypersensitive response in the infected leaves. Moss was grown under the following conditions: A) negative control: BCD, uninfected; B) positive control: BCD, infected; C) BCD + 1 mM LaNO$_3$, infected; D) BCD + 50 mM D600, infected; E) BCD + 5 mM EGTA, infected; F) BCD + 1 mM LaNO$_3$ + 50 mM D600 + 5 mM EGTA, infected. Purple staining represents fortifying modifications to cell wall polysaccharides and blue staining represents the production of phenolics. *P. irregulare* is noted with an arrow and congregating chloroplasts are noted with a box.
Diaminobenzidine (DAB) staining was used to investigate the production of ROS such as H₂O₂ in response to infection. Red/brown staining represents the presence of H₂O₂. All moss treated with inhibitors and exposed to *P. irregulare* appeared to show increased production of H₂O₂ as compared to the BCD control (Figure 4).

**Figure 4.** DAB staining of infected *M. cuspidatum* treated with calcium inhibitors. *M. cuspidatum* was exposed to *P. irregulare* for 24 hrs then stained with DAB to observe the production of the ROS H₂O₂ in response to infection. Moss was grown under the following conditions: **A)** negative control: BCD, uninfected; **B)** positive control: BCD, infected; **C)** BCD + LaNO₃, infected; **D)** BCD + D600, infected; **E)** BCD + EGTA, infected; **F)** BCD + 1 mM LaNO₃ + 50 mM D600 + 5 mM EGTA, infected. Brown/red staining indicates the presence of H₂O₂.
Autofluorescence was used to investigate the autofluorescence of chloroplasts and phenolics in healthy leaf cells vs. infected leaf cells. Moss was grown under all 4 aforementioned inhibitor treatments as well as under a BCD control treatment for 24 hours. Normal chloroplast autofluorescence and was observed in uninfected moss grown on the BCD negative control, while chloroplast and subsequent cell death was evident in infected moss treated with the inhibitors. Phenolics did not observably autofluoresce in healthy, uninfected cells grown on the BCD negative control, but produced substantial observable autofluorescence in moss grown on all inhibitor treatments (Figure 5). Infected moss grown on calcium inhibitors versus infected moss grown on the BCD positive control showed decreased autofluorescence of chloroplasts and increased autofluorescence of phenolics in response to inoculation of *P. irregulare*.

Additionally, light microscopy was used to investigate the presence of chloroplast congregation that is typical in plants exposed to infection or other stressors. Normal chloroplast distribution was observed in uninfected cells, but the opposite is true for moss exposed to fungus and grown on inhibitor treatments (Figure 3D).
Figure 5. Autofluorescence of chloroplasts and phenolics in *M. cuspidatum* in response to infection with *P. irregulare*. Red shows autofluorescence of healthy chloroplasts while blue shows autofluorescence phenolics in response to the following treatments: A, B) BCD negative control, uninfected; C, D) BCD positive control, infected; E, F) BCD + 1 mM LaNO$_3$ + 50 mM D600 + 5 mM EGTA; … (continued on next page).
Figure 5 (continued). Autofluorescence of chloroplasts and phenolics in *M. cuspidatum* in response to infection with *P. irregulare*. Red shows autofluorescence of healthy chloroplasts while blue shows autofluorescence phenolics in response to the following treatments: (continued from previous paged) **G, H** BCD + 1 mM LaNO$_3$; **I, J** BCD + 50 mM D600; **K, L** BCD + 5 mM EGTA. All images were made on a Leica DMLB compound fluorescent microscope in conjunction with an ExiQ imaging camera and Metamorph v7.78 software.
**Discussion**

Mosses, especially *Mnium cuspidatum*, are nonvascular and considered to be lower plants, and are therefore oftentimes disregarded in research. Thus, little is known about their defense responses and the specific chemicals within such mechanisms. Previous studies regarding higher, vascular plants have shown that calcium acts as an important second messenger in initial defense responses to pathogens (Zhang et al, 2014). However, it is unknown to what extent calcium in defense mechanisms in moss. Upon inhibiting the extracellular and intracellular flow of calcium in the moss *M. cuspidatum*, a qualitative decrease in defense mechanisms and defense chemical production was observed, thus suggesting that calcium is in fact a conserved component of defense mechanisms across the plant kingdom.

**Calcium Is Involved in Specimen Growth**

In the current study, the role of calcium in the growth of moss *M. cuspidatum* and fungus *Pythium irregulare* was initially investigated in order to ensure that each would still grow in the presence of calcium inhibitors. Differences in growth were observed among the moss controls versus the moss grown on inhibitor treatments (1 mM LaNO₃, 50 mM D600, 5 mM EGTA), though these differences were not significant. The EGTA treatment for both *M. cuspidatum* and *P. irregulare* fungus was originally 500 mM, but this totally inhibited growth of the fungus. EGTA treatment was then reduced to 5 mM and *P. irregulare* was able to grow, though exact data was not obtained. No significant differences in growth of control fungus versus fungus grown on the remaining inhibitor treatments (1 mM LaNO₃, 50 mM D600) were found.
Jackson and Heath (1993) describe the many roles of calcium in the growth of fungal hyphae, and state that growth ceased in hyphae devoid of calcium. Moreover, hyphae growth has been determined to be dependent on a specific free calcium gradient (100-350 nM). This leads me to believe that the initial EGTA concentration of 500 mM was far too great to remain within that necessary free calcium gradient. That is, it is likely that this EGTA treatment completely chelated extracellular calcium in the PDA medium on which *P. irregulare* was grown.

**Calcium Mediates Hypersensitive Response**

Qualitative analysis using various forms of microscopy suggests that abundance of defense molecules such as H$_2$O$_2$, phenolics, and polysaccharides is altered in the presence of some calcium inhibitors. Moss grown on 1 mM LaNO$_3$ then infected with *P. irregulare* did not appear to alter defense chemical production (Figures 3C and 4C). This suggests one of two things: 1) the concentration of LaNO$_3$ was too small to have any effect and needs to be increased to obtain valid results or, 2) LaNO$_3$, thought to inhibit the activity of non-selective (but calcium permeable) cation channels that usually compensate for changes in calcium (Demidchik et al, 2002), does not have any effect on *M. cuspidatum* calcium uptake.

Moss grown on 50 mM D600, a derivative of verapamil that acts as a calcium ion channel antagonist in plasma membranes (Singh et al, 2010), then infected appeared to exhibit less production of the ROS chemical H$_2$O$_2$ (Figure 4D) but the same phenolics and polysaccharide abundance and pattern (Figure 3D). This apparent decrease in H$_2$O$_2$ production may suggest that calcium mediates defense mechanisms at the point at which
these ROS are generated and/or released. That is, without adequate calcium in the cell, production of ROS may be inhibited. Again, concentration of D600 should be altered to further investigate they hypothesis and validate these results.

Moss grown on 5 mM EGTA, an extracellular calcium chelator, then infected exhibited a substantial decrease in H$_2$O$_2$ production as compared to the BCD positive control (Figure 4E). This may suggest that the removal of calcium in the medium that ultimately prevents its uptake also prevent the adequate sequestering of calcium to produce and/or release ROS. Toludine blue staining of moss exposed to this treatment exhibited the same abundance and pattern of phenolic and polysaccharide production as compared to the BCD positive control (Figure 3E).

Finally, *M. cuspidatum* grown on all three inhibitors at the same time then inoculated with *P. irregulare* seem to exhibit the same degree of H$_2$O$_2$ production as compared to the BCD positive control (Figure 4F). The same treatment seemed to elicit a more widespread phenolic and polysaccharide production (Figure 3F), perhaps suggesting a quicker induction of HR. This may also suggest that increased inhibition of calcium in the moss prevented initial responses such that the moss needed additional, more widespread chemical responses for HR.

Regardless of the severity of the HR response, it seems to present itself in a gradient of sorts, particularly in the BCD positive control treatment (Figure 4B) as well as in each of the other single-inhibitor treatments (Figure 4C-E). Using toluidine blue staining, one can observe this gradient to be characterized healthy cells farthest away from sight of infection to chloroplast congregation, phenolic production, and polysaccharide production in cells increasingly close to the sight of infection. This
suggests that phenolics are associated with early-phase HR and polysaccharides are associated with later-phase HR.

Analysis of autofluorescence of chloroplasts and phenolics in *M. cuspidatum* following infection with *P. irregulare* revealed that moss grown on calcium inhibitors then infected with fungus present with increased cell death (noted by chloroplast autofluorescence) and increased cell wall fortification (noted by phenolic autofluorescence) in comparison to infected moss grown on inhibitor-free medium (Figure 5 C-L). However, there is no obvious difference in this autofluorescence among inhibitor treatments. Further investigation into this might include quantification of autofluorescence to determine if any differences are statistically significant.

In order to determine if there is an association between the amount of calcium and the apparent gradient or progression of HR, one could tag calcium with EGTA-based fluorescent indicators. Two main types of fluorescent calcium indicators exist to determine the distribution of cytoplasmic free calcium: ratio dyes and single-wavelength dyes. Ratio dyes are based on the premise that bound calcium and free calcium fluoresce at different wavelengths. Thus, these dyes enable one to distinguish and measure the difference in intensity of the fluorescence of bound calcium and free calcium. Single-wavelength dyes, on the other hand, exhibit changes in fluorescence intensity when bound to calcium. Thus, regions with more accumulation of calcium with fluoresce more, enabling one to detect a gradient of calcium within the cell (Jackson and Heath, 1993). With such techniques, it may be possible to determine the exact location of calcium within the cells of *M. cuspidatum* and not only give an exact location to the inhibition of calcium, but to also determine whether or not a gradient in HR is related to a gradient in
calcium abundance. That is, fluorescently tagging calcium may help in determining if there is an association between abundance of calcium and the extent of HR.

Another method used to determine the location of calcium in the cell is by dying specimens with Chlortetracycline (CTC), a fluorescent dye that indicates the presence of calcium in the vicinity of a membrane (Jackson and Heath, 1993). CTC assays may be beneficial in future aspects of this study to determine the exact role of calcium in defense mechanisms and whether it mediates defense mechanisms such as those associated with fortifying the cell wall and cell membrane. It may also be beneficial in observing whether or not calcium localizes to vesicles and organelles during defense mechanisms or if it is instead released by certain organelles as a byproduct of reactions. Such assays would aid in determining the exact mechanism by which calcium mediates defense mechanisms in *M. cuspidatum*.

**Calcium as a Potential Conserved Component in Defense Mechanisms**

Though the present study does not provide completely conclusive results regarding the role of calcium in HR in *M. cuspidatum*, it is apparent that it mediates some aspects of the defense response. Calcium has been noted to act as a second messenger in many signal transduction pathways including those involved in stress response (Tuteja, 2007). Specifically, changes in calcium concentration have been directly associated with ROS production and defense gene expression in tobacco plants (Zhang et al, 2014). Calcium has also been described as being the initial trigger of wound inflammatory response in humans (Razzell et al, 2013). Thus, further investigation of the role of
calcium in moss defense responses is warranted in order to understand the potential conservation of such mechanisms.

Future studies could include polymerase chain reactions (PCR) techniques to determine relative expression of the defense genes PAL, PR-4, and PR-5 in *M. cuspidatum* grown on calcium inhibitors and infected with *P. irregulare* (Fawcett, 2014). A decrease in expression of these genes might suggest that calcium mediates signal transduction pathways that control their expression.
Conclusion

Using the moss *Mnium cuspidatum* and the fungal pathogen *Pythium irregulare*, this study investigated the role of calcium in defense responses, specifically hypersensitive response (HR). Though results were not completely conclusive, some notable observations were made. Toludine blue staining showed an apparent wider spread of production of cell-wall fortifying polysaccharides and phenolics in moss grown with a treatment of 1mM LaNO₃, 50 mM D600, and 5 mM EGTA then infected with fungus. With DAB staining techniques, an apparent decrease in ROS production in response to infection was observed in moss grown with 50 mM D600 as well as with 5 mM EGTA. Autofluorescence of healthy chloroplasts was less intense and dense in infected moss treated with calcium inhibitors, and autofluorescence of cell wall phenolics were denser in these same specimens. Though further investigation is necessary, these findings provide initial support for the hypothesis that calcium mediates defense responses in the moss *M. cuspidatum*. 
References


