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Role of 12-oxo-phytodienoic acid and β-glucan Elicitor in Prevention of Fungal Infection in Mnium cuspidatum

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Role of 12-oxo-phytodienoic acid and β-Glucan Elicitor in Prevention of Fungal Infection in *Mnium cuspidatum*

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ABSTRACT

The moss, *Mnium cuspidatum* and the fungus, *Pythium irregulare,* were used to study the role of the plant hormone, 12-oxo-phytodienoic acid (OPDA) and the elicitor, β-glucan in the prevention of fungal infection. Each compound was applied before the inoculation of a fungal cube and a combination of qualitative and quantitative analyses were done in order to determine the degree of infection within the moss. It was found that *P. irregulare* does infect and kill the cells of *M. cuspidatum* through chloroplast degradation, stem browning and appressoria injections within 24 hours. The pre-application of OPDA or β-glucan were unable to prevent or lessen the fungal infection. In future experiments, it is recommended that a cellophane cover be applied before the application of the moss and fungi so that the fungi does not grow into the medium and the moss does not soften from contact with the agar gel.

INTRODUCTION

All organisms must respond to their environment in order to survive and reproduce. One primary example of this occurs in host-pathogen interactions. Pathogens are considered to be any disease-producing agent, especially a virus, bacterium or other microorganism. Typically, they infect the host so they can benefit from it in some way. Pathogens have developed numerous mechanisms in order to invade the host and break down its external barriers. For example, fungi have specific enzymes used to break down the cell walls of plants so that they can obtain nutrients from them (Ponce de Leon *et. al*, 2007).

Moss-fungi interactions and defense mechanisms of plants

In this study, a fungus (*Pythium irregulare*) was chosen as the pathogen and moss (*Mnium cuspidatum)* was used as the host organism. Mosses are non-vascular plants that are the most primitive of any land plant and are idyllic for studying biological systems in plants because they appear to employ similar defense mechanisms to the most recently derived flowering plants (Lang *et. al* 2008; Oliver, 2009). It has previously been shown that numerous fungi are capable of infecting the moss, *Physcomitrella patens,* causing browning, chloroplasts degradation and cytoplasmic collapse. More specifically, chloroplasts have been shown to orient themselves towards the site of infection (Ponce de Leon *et. al*, 2007).

Plants have developed responses in order to combat pathogens. They do this in two primary ways: hypersensitive response (HR) and systemic acquired resistance (SAR). Hypersensitivity is a mechanism that the plant uses in order to stop the spread of a pathogen. It does this by quarantining the pathogen from the rest of the plant. The plant

produces cellulose in infected cells in order to strengthen the cell walls and effectively sequester the pathogen. The plant then kills healthy cells around the area of infection by apoptosis. This process isolates the pathogen and prevents it from spreading to other areas of the plant. Hypersensitivity is a quick, localized response to the pathogen and usually initiates the SAR response (Ponce de Leon *et. al*, 2007).

The SAR response is systemic and it involves the production of specific compounds within the plant such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and OPDA (Glazebrook, 2005). OPDA and ET are typically made when the pathogen is a necrotroph, such as some fungi. The presence of these hormones initiates a defense response in the plant that is used to prevent future pathogen infections and lessen their effects within the plant.

Roles and relationship between JA and OPDA

It was previously thought that the jasmonic acid itself was indirectly responsible for the inhibition of fungi growth on mosses by initiating chemical defenses. However, further research has indicated that the precursors of the jasmonic acid molecule can also initiate plant defenses. For example, the OPDA molecule is a biologically active precursor that is made during the metabolic pathway used to form the jasmonic acid molecule. Its application alone has been shown to inhibit future pathogen infection by initiating the SAR response (Turner et al, 2002).

Life cycle of M. cuspidatum

Mnium cuspidatum is a species in the Bryophyta division, Bryopsida class, Bryales order and Mniaceae family (Montana Field Guide). Mosses belonging to the Class Bryidae are considered to be "true mosses." All true mosses have multicellular rhizoids, which are important in support. Mosses also have rudimentary stems and leaves for functional support and photosynthesis.

The life cycle of a moss consists of both haploid and diploid stages (Figure 1). In the haploid stage, the moss has only one set of chromosomes whereas in the diploid stage, the moss has two sets of chromosomes. Spores are released as haploids and grow into young male and female gametophytes (buds). When they are more mature, sperm are released from the antheridial head of the male gametophytes and transferred to the archegonial head of the female gametophyte through water. The sperm are chemically attracted to the haploid egg within the neck canal of the female gametophyte. The sperm fertilizes the egg and a diploid zygote grows into an embryo. The mitotically dividing zygote becomes the sporophyte, which is encased in the calyptra. Sporophytes consist of a capsule called the sporangium, which is supported by the seta. Meiosis occurs within the sporangium and spores are once again released after the operculum (small lid of the sporangium) opens (Raven, Evert and Eichhorn, 2005).

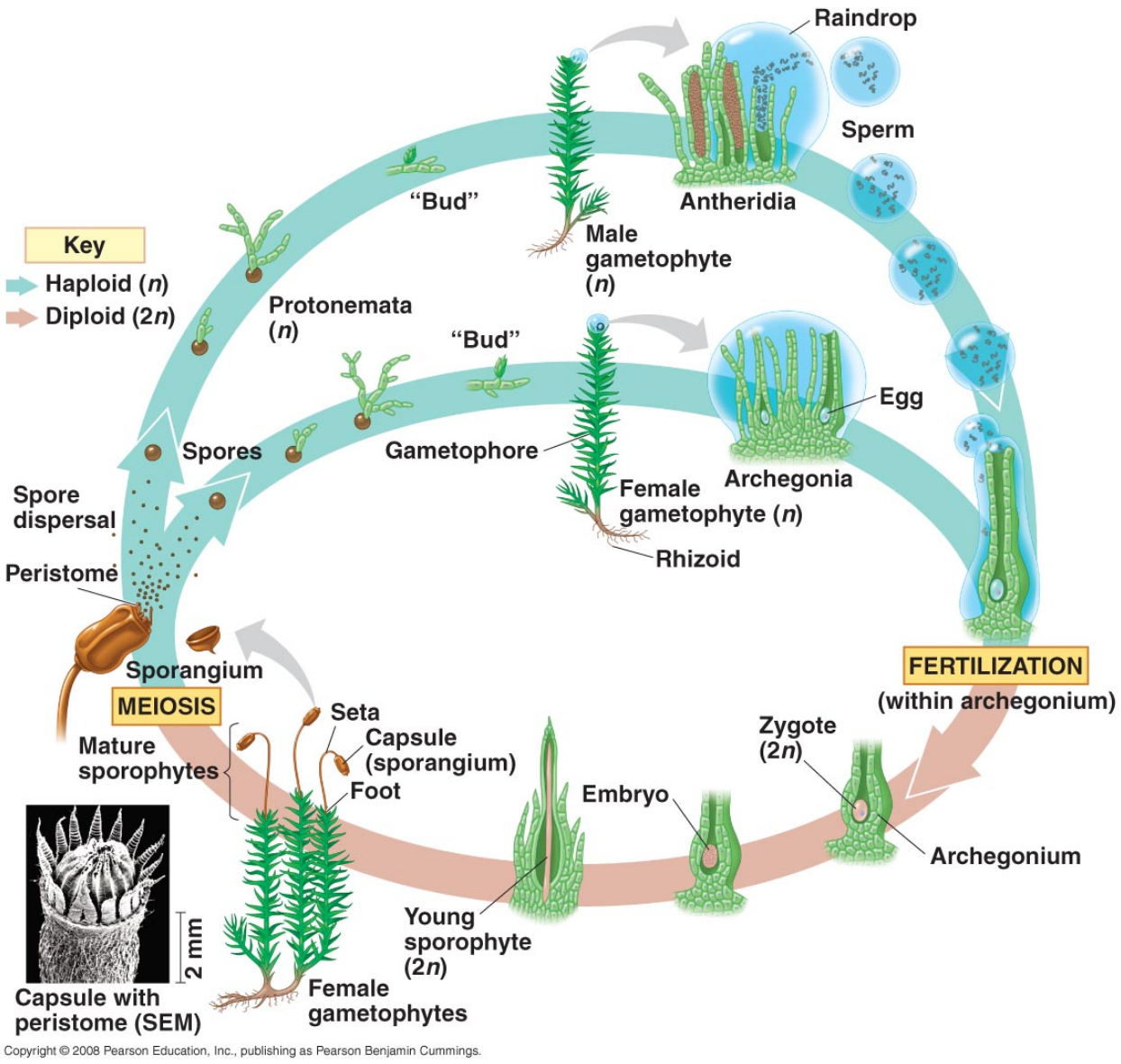

Figure 1. The typical life cycle of a moss. (Campbell and Reece, 2005).

Life cycle of P. irregulare

P. irregulare is a soilborne pathogen that is known to infect hundreds of plants worldwide. It is an oomocyte in the Pythiaceae family and is typically characterized by hyphae

5 µm in diameter and spherical sporangia. The species has both sexual and asexual stages which both result in infection by the hyphal germ tubes (Katawczik, 2008).

In the life cycles of oomycetes, asexual reproduction is primarily used (Figure 2). In asexual reproduction, the zoosporangium encases biflagellated zoospores, which are released and subsequently develop into an encysted zoospore. The encysted zoospore then develops into a secondary zoospore with lateral flagella. The secondary zoospore encysts once again and then germinates to produce a new mycelium. A mycelium is a mass of hyphae forming the body of a fungus or oomycete. During sexual reproduction, oogonia and antheridia form on the hyphae and meiosis occurs within the oogonia to produce haploid eggs. The antheridia grow towards the oogonia and penetrate the oogonia with fertilization tubes. Post fertilization, a zygote, or oospore, is produced. This structure then develops into a hypha, producing a zoosporangium and the cycle is continued (Raven, Evert and Eichhorn, 2005).

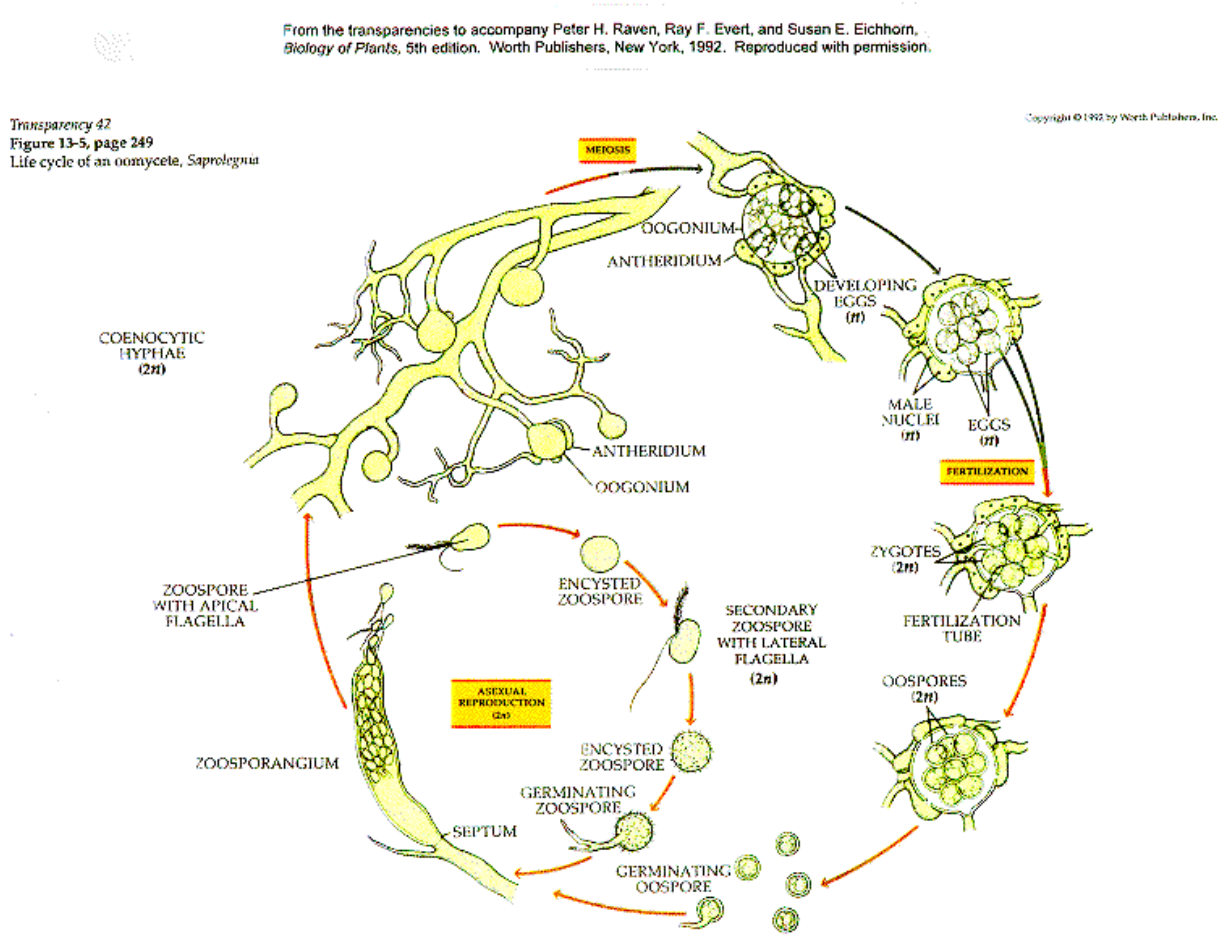

Figure 2. The typical life cycle of an oomycete. (Raven, Evert and Eichhorn, 2005).

Choice of M. cuspidatum and P. irregulare

There has been no research investigating the roles of OPDA or other jasmonates on *M. cuspidatum*, which is the primary reason that this moss was chosen for the experiment. Another reason that the moss was chosen was that it can be quickly propagated on an artificial medium.

P. irregulare was chosen to be the pathogen because it has a broad host range and it can be easily grown on an artificial medium. It was also chosen because two other studies that

have investigated non-vascular plant defense mechanisms used this pathogen (Bowman, 2011; Oliver *et. al*, 2009).

Appressoria and access to plant cell

Dome-shaped penetration pegs known as appressoria are the primary way that fungi invade the cell wall and gain access to the inner contents of the plant (Davey *et. al*, 2009). They do this by producing hydrolytic enzymes that weaken or break down the cell wall. With hydrostatic pressure, the infection peg penetrates the cell wall and the hypha can gain access to the contents of the cell (Ponce de Leon *et. al*, 2007).

Function of elicitors in plant response

When a plant is attacked by a specific pathogen, the plant normally recognizes the characteristics and structure of it. Once the plant does this, the defense responses within the plant are elicited and signals are sent throughout it. For example, after fungal identification, pathways for JA or OPDA production can be started and the plant can begin its systemic response.

An elicitor is any compound that stimulates any type of plant defense. Elicitors can be biotic or abiotic. Biotic elicitors have a biological origin, derived from the pathogen or the plant itself while abiotic elicitors have no biological origin and are grouped as physical factors or chemical compounds. When applying an elicitor to the plant, the plant elicits the same response as it would if the whole pathogen was attacking it even though the elicitor does not have the same detrimental effects like a pathogen would (Patel and Krishnamurthy, 2013). In

this study, β-glucan was used as a biotic elicitor because it is part of the cell wall of *Pythium* fungi and is recognized by *M. cuspidatum.*

Previous experiments have used fungal elicitors in order to elicit a defense response in plants. Ponce de Leon *et. al* (2007) found that necrotrophic fungi have the capability to induce a defense response in the moss, *Physcomitrella patens,* by causing enhanced expression of defense-related genes. Similarly, Lehtonen *et. al* (2009) discovered that chitosan, a derivative of some fungal cell walls, can successfully induce a defense response in *P. patens.*

Goals and Objectives

There were three parts to this study. The primary aim of part one was to determine if OPDA alone could hinder or prevent the growth of the fungus, *P. irregulare.* The second part focused on the role of OPDA in preventing fungal growth on the moss, *M. cuspidatum.* Part three aimed to determine whether the elicitor, β-glucan, could help prevent or lessen the infection of fungi on the moss.

Thesis hypotheses

I hypothesize that the fungi, *P. irregulare,* will infect the moss, *Mnium cuspidatum* through observation of appressoria formation, moss chloroplast degradation and stem browning. However, if OPDA is previously applied to the moss, then the *M. cuspidatum* will be healthier than it would if OPDA were not pre-applied. I also hypothesize that the β-glucan elicitor will stimulate the SAR response and the fungi will have a lesser effect on the moss. This

research will expand the understanding of OPDA in the defense response of mosses and add to the knowledge of pathogen interactions between fungi and non-vascular plants.

MATERIALS AND METHODS

Growing Mnium cuspidatum and P. irregulare

Living *M. cuspidatum,* grown in a BCD medium, were taken from the lab of Dr. Phillip Villani at Butler University Lab and used in each part of the study. They were grown aseptically on a medium containing 1.0L of BCD medium, 10 mL each of solutions B, C, D, 8.0 g agar, 920 mg di-ammonium (+) tartrate and 800 mL of distilled water at a pH of 6.5. Stock solution B consisted of 25 g of $MgSO_4$ in 1 L distilled H_20. Stock solution C consisted of 25 g of KH_2PO_4 in 500 mL of distilled H_20. Stock solution D consisted of 101 g KNO_3 and 1.25 g $FeSO_4$ in 1 L of distilled H_20. The solutions were mixed and brought to a boil in a microwave. Next, 4 g of agar gel was added and the solution was autoclaved. After the autoclave, 1 mL of 1 M $CaCl_2$ was added and the solution was then poured into petri dishes. The moss was grown at 25^{o}C under fluorescent lighting and was exposed to light 16 hours a day. Moss used for experimentation was taken from previously growing cultures that were between 3-5 months old. Flame sterilized instruments and a laminar airflow hood was used to transfer the moss from dish to dish.

A 0.25x0.25 cm cube of previously growing *P. irregulare* was taken from a 3 month old plate in the Butler University Lab and placed on a new plate from which experimentation was done. The fungus was grown on a PDA medium made from mixing 9.75 g potato glucose agar and 3.75 g Difco agar. Next, 500 mL of water was added and the solution was autoclaved and

poured into sterilized petri dishes. Flame sterilized instruments and a laminar airflow hood were used to transfer fungal cubes from the PDA medium to the moss tips.

Solutions of OPDA and β-glucan

The OPDA was taken from a bottle consisting of 100 µg dissolved in 100 µL EtOH (3.42 mM). The solution was then diluted to 50 µM by adding 3.32 mL ethanol and 3.52 mL of distilled water. The 50 µM solution was used as the working solution in all cases where OPDA was used. β-glucan was diluted to 1mg/mL; the 1mg/mL dilution was the working solution in all cases where β-glucan was used.

Fungal infection of moss

In order to determine if OPDA could be used to prevent infection of *P. irregulare,* it had to be shown that the fungus was capable of infecting and killing the moss, *M. cuspidatum*. Cubes of 0.25x0.25 cm *P. irregulare* were taken from a week old petri dish and placed onto the tip of a 2 cm piece of *M. cuspidatum* in a quarter plate with a BCD medium. Half of the moss was placed on the medium while half was hanging off of the other side of the divide. The fungal cube was placed on the side opposite the medium to ensure that it would only infect the moss and not grow within the medium. The fungus was allowed to infect the moss for 24 hours, after which it was inspected under an Olympus, CHK2-F3-100 model microscope and stained with a 0.01% solution of lacto-phenol trypan blue. The trypan blue was used because it only stains the inside of the fungi and not the moss, making it much easier to see parts of infection. Two pieces of moss were placed on each plate (one on each side of quarter plate) and two plates were considered to be one trial. In all, two trials were done in order to determine if

the fungi could adequately infect the moss. Chloroplast degradation, fungal appressoria formation, and stem browning were the primary signs that infection had occurred.

Part one- Can OPDA directly inhibit fungal growth?

OPDA and its solvent were used in this part of the experiment. It had to be shown that OPDA alone could not kill the fungi. In order to ensure this, three cubes of 0.25x0.25 *P. irregulare* were placed onto three separate 1/8 PDA plates. After 24 hours, 15 µL OPDA and its solvent (ethanol + water) were applied approximately 2 cm from the growth front of the fungi. On the second plate, 15 µL of the solvent alone was placed approximately 2 cm from the growth front of the fungi. There were no compounds or solvent added to the third plate and it was used as a control for the other two plates. The infection spots of the OPDA and solvent were marked on the bottom underside of the plate as a method for analysis. The three groups of plates with OPDA + solvent, solvent only and no solvent or OPDA were considered one trial and in total, 3 trials were done. All of the trials were checked after 48 hours and measured from the distal tip (nearest point of fungi to farthest side of plate) to the farthest point of the growth front. The second trial was measured after 24 hours and the third trial was measured after 36 hours.

Part two- Does OPDA pretreatment protect the moss from fungal infection?

In this part of the experiment, moss samples were treated with OPDA prior to inoculation in order to determine if it's pre-application could prevent or lessen the effects of the fungi. Fungal cubes were applied at either the distal end (DE) or proximal end (PE) to determine if the response signal was transmitted throughout the plant. A quarter plate with

BCD medium was used for all trials; there were two control and one experimental plate per trial. In total, four trials were done.

Control one consisted of two pieces of moss (one on each side of quarter plate) with one piece having its distal end in the medium and the other piece with its proximal end in the medium. Approximately one half of the 2 cm piece of moss was placed on top of the medium while the other half was hanging off from the divide (Figure 3). Immediately after the moss was placed onto the plate, 15 µL of solvent (ethanol + water) was placed onto the tip on top of the medium. Nothing more was added to the moss and it was allowed to grow for 7 days.

Figure 3. Moss hanging off quarter plate. Piece of *M. cuspidatum* with one half in BCD medium (left) and one half hanging off from quarter plate divide (right).

Control plate two was done in the exact same way as control one except that a 0.25x0.25 cm fungal cube was added to the end opposite the medium approximately 8 hours

after the application of the solvent. The plate was then allowed to grow for 7 days until inspection.

The experimental plate was done with the same method as control two except that 15 µL of OPDA was applied to the tip on top of the medium instead of the solvent. The fungal cube was applied approximately 8 hours after the application of the OPDA, as previously stated and the fungi was allowed to grow for 7 days until inspection.

After 7 days, each piece of moss was inspected under an Olympus, CHK2-F3-100 model microscope. The moss was stained with 0.01% lacto-phenol trypan blue and chloroplast degradation, appressoria and stem browning were signs that were searched for to determine if infection occurred.

To determine if an area of the moss was infected/dead, one or more of the following characteristics had to be observed. If 50% or more of the chloroplasts within a leaf had been displaced or degraded, then it was considered that the leaf and the adjacent part of the stem were dead. If the stem was completely brown or translucent, then it was determined that the area was dead. If an area had multiple blue, stained fungal hairs surrounding it and appressoria were found, then it was determined that the section of the moss was dead.

Part three- Does β-glucan pretreatment protect the moss from fungal infection?

The goal of this part of the experiment was to determine if pretreatment of β-glucan on the moss could prevent or lessen fungal infection. One trial consisted of one control plate, one

experimental plate and one plate that had both an experimental and a control piece of moss. Four trials were done in total and quarter plates with BCD medium were used.

In the control plate, approximately one half of a 2 cm piece of moss was placed on the medium with the other half hanging off the edge of the divide. Next, 30 µL of β-glucan was applied to the tip on the medium. On the other side of the plate, a piece of moss was placed on the medium as previously stated and 30 µL of sterile water was placed on the tip on the medium. Nothing more was added to pieces and they were allowed to grow for 7 days.

In the control and experimental plate, approximately one half of a 4 cm piece of moss was placed on top of the medium with the other half hanging off. Since the piece of moss was so long, the end opposite the medium touched the bottom of the dish. After the piece was placed on the medium, 15 µL of β-glucan was applied to the piece every 0.5 cm. After 24 hours, a cube of 0.25x0.25 fungi was added to the end opposite the medium. In two trials, the DE was placed on the medium and in the other two trials, the PE was placed on the medium. On the other side of the plate, a 2 cm control piece of moss was placed across the divide, with half of it on the medium and the other half hanging off. Nothing was added to the piece. Both the control and experimental pieces were allowed to grow for 7 days.

In the experimental plate, one 2 cm piece of moss was placed on the medium with half of the piece hanging off the divide and the other on top of the medium. In half of the trials, the DE was placed on the medium and in the other half, the PE was placed on the medium. After the moss was placed on the plate, 30 µL of β-glucan was added to the tip on top of the medium.

After 24 hours, a 0.25x0.25 fungal cube was added to the end opposite the medium. The moss was allowed to grow for 7 days.

After 7 days, each piece of moss was inspected under an Olympus, CHK2-F3-100 model microscope. The moss was stained with 0.01% lacto-phenol trypan blue and chloroplast degradation, appressoria and stem browning were signs that were searched for to determine if infection occurred.

Statistical analysis

A two-tail t-test was done in order to determine the statistical significance of the results. The average of the control two trial from part two (ethanol and water inoculated with fungus) was used as the hypothetical mean because it could be used as a baseline in order to see if other experimental trials with OPDA and β-glucan were able to prevent or lessen infection. If the OPDA or elicitor "worked" and prevented or lessened infection, then the results would be different from the control two trial of part two. If the p-value was less than 0.05, then it indicated that the trial had results that were different from the control two plates and the OPDA prevented or lessened infection. If the p-value was greater than 0.05 then it was concluded that the trial was similar to the control two plate and the experimental trial did not prevent or lessen infection.

RESULTS

Fungal infection of moss

After 24 hours, the moss samples were examined under an Olympus, CHK2-F3-100 model microscope. Chloroplast degradation was observed in all eight pieces of moss, as well as stem browning, appressoria and stained, blue fungal hairs (Figure 4). After consideration, it was determined that all eight pieces were infected.

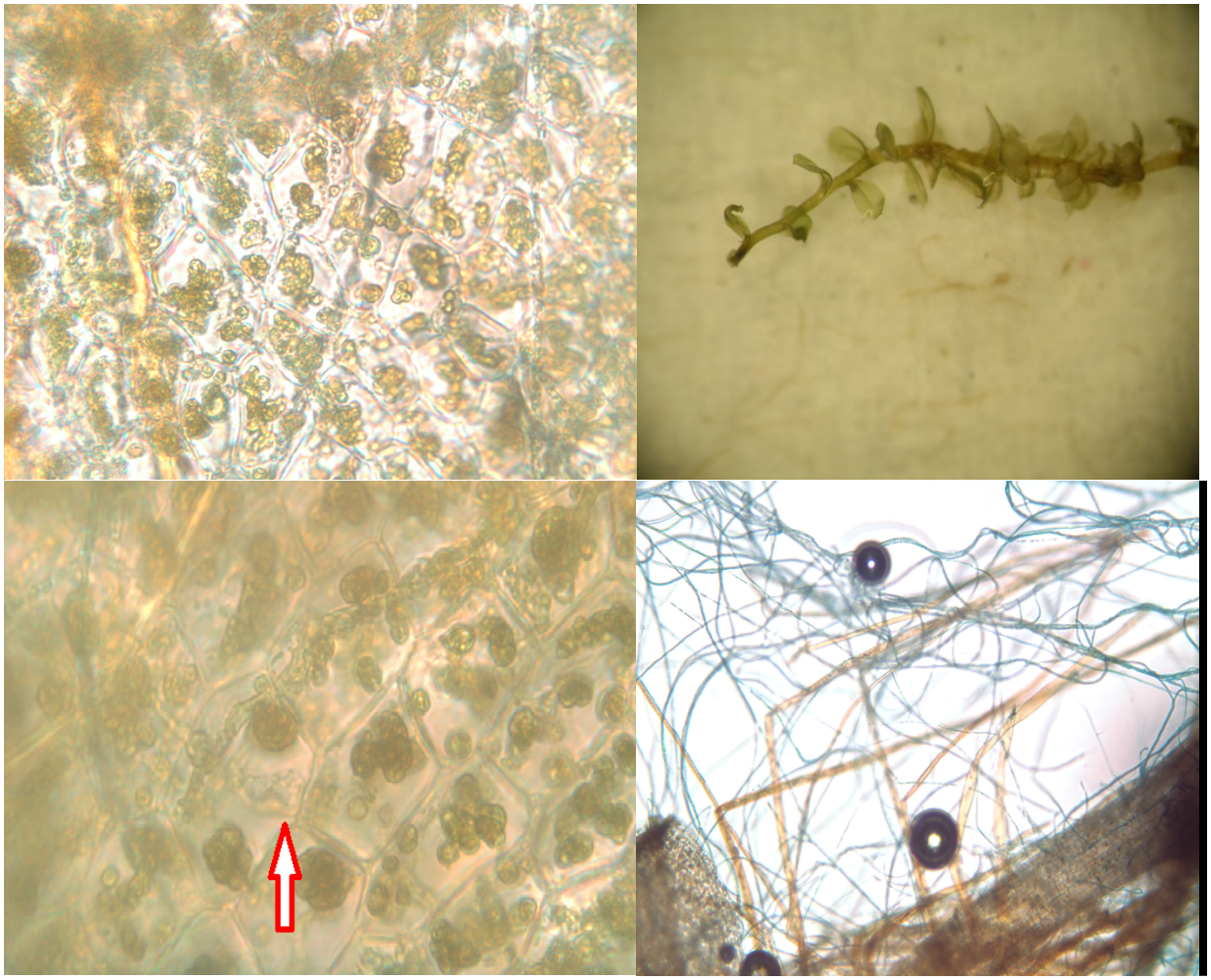

Figure 4. Pythium infection on *M. cuspidatum*. Four characteristics were observed in order to determine if the section of moss was infected/dead: chloroplast degradation or displacement (top left and bottom left), stem browning (top right), appressoria (bottom left) and stained, blue fungal hairs (bottom right).

Part one- Can OPDA directly inhibit fungal growth?

The three groups of plates with OPDA + solvent, solvent only and no solvent or OPDA were considered one trial and in total, 3 trials were done. Each plate of the three trials was measured after 48 hours. Trial two was also measured after 24 hours and trial three was measured after 36 hours. In all three trials, each plate was completely infected with fungi after 48 hours. The distance from the distal tip of the fungi to the opposite end of the plate (farthest point of plate from fungi) varied for each trial (Tables 1 and 2).

Table 1. Infection of fungi on plate after 48 hours. Each plate from part one was measured from the distal tip of the fungi to the opposite end (farthest away) of the plate after 48 hours.

PLATE	TRIAL	DISTANCE	Percentage of infection
Control- only fungi	1	7.2 cm	100
Control- solvent	1	7.0 cm	100
Experimental-OPDA	1	7.4 cm	100
Control- only fungi	2	6.8 cm	100
Control- solvent	2	6.7 cm	100
Experimental-OPDA	2	6.9 cm	100
Control- only fungi	3	6.2 cm	100
Control- solvent	3	6.1 cm	100
Experimental-OPDA	3	5.9 cm	100

Table 2. Infection of fungi on plate after 24 and 36 hours. The plates from trial two were measured from the distal tip of the fungi to the farthest point of the growth after 24 hours. The same measurement was done for trial three after 36 hours.

PLATE	TIME	TRIAL	DISTANCE	Percentage of infection
Control- only fungi	24	2	3.1 cm	45
Control- solvent	24	2	3.0 cm	45
Experimental-OPDA	24	2	3.1 cm	45
Control- only fungi	36	3	5.8 cm	94
Control- solvent	36	3	5.6 cm	92
Experimental-OPDA	36	3	5.9 cm	100

Part two- Does OPDA pretreatment protect the moss from fungal infection?

Control one consisted of two pieces of moss (one on each side of quarter plate) with one piece having its distal end in the medium and the other piece with its proximal end in the medium. Immediately after the moss was placed onto the plate, 15 µL of solvent (ethanol + water) was placed onto the tip on top of the medium. Control plate two was done in the exact same way as control one except that a 0.25x0.25 cm fungal cube was added to the end opposite the medium approximately 8 hours after the application of the solvent. The experimental plate was done with the same method as control two except that 15 µL of OPDA was applied to the tip on top of the medium instead of the solvent. The fungal cube was applied approximately 8 hours after the application of the OPDA, as previously stated and the fungi was allowed to grow for 7 days until inspection.

Daily, qualitative observations were made on each plate of the four trials to monitor the progress of the infection or lack thereof. In some cases, the moss was unable to be observed because it was completely disintegrated (CD) or partially disintegrated (PD) within the medium (Table 3). In these cases, it was determined that the appressoria had ruptured the cell walls and the moss was 100% dead. Pictures were taken at day seven to provide a qualitative comparison (Figure 5).

Figure 5. Control one, control two and experimental moss. Side-by-side comparison of control one (left), control two (center) and experimental (right) moss. The fungal cube can be seen at the left end of the moss (center).

While most of the daily examinations just indicated a steady progression of the infection of the moss, there were some noteworthy observations. After the first inspection (24 hours after application of fungi), there did not appear to be much, if any, fungal growth on the experimental plates. However, in all pieces of moss except one, there was some indication of fungal growth on the control two plates. It was also noted that all of the fungal infection had occurred by day 4-5. After these days, the amount of fungal infection did not appear to change. The final noteworthy observation was that the fungus crossed over from the moss and infected the BCD medium.

The mean percentage of infection for the experimental, DE trials was 82.5% and 95% for the experimental, PE trials. The mean percentage of infection for control one, DE trials was 21.25% and 48.75% for the control one, PE trials. The mean percentage of infection for the

control two, DE trials was 95% and 87.5% for the control two, PE trials (Figure 6).

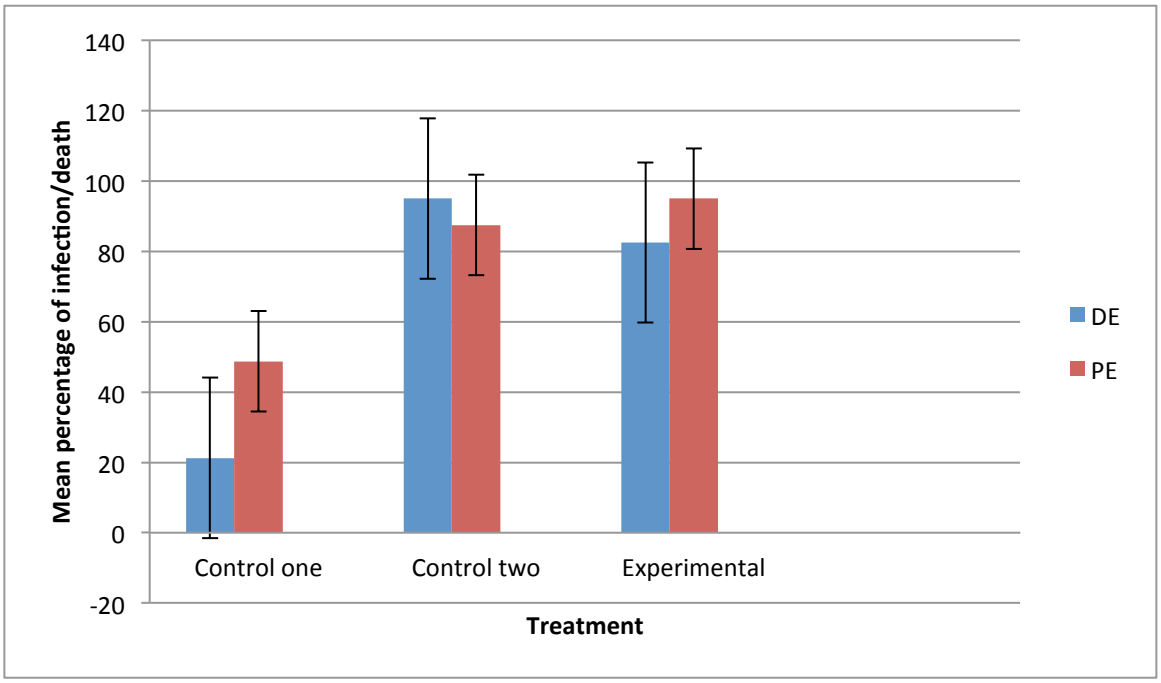

Figure 6. **Infections of control one, control two and experimental trials.** The mean percentages of infection were calculated by examining the moss for appressorial injection, stem browning, chloroplast degradation and blue fungal hairs. Each piece of moss was individually observed and the percentage of the dead areas of the piece was determined. Next, the average of the dead areas of each piece was calculated and standard error bars were inserted. Control one plates were treated with 15 µL of solvent (ethanol + water). Control plates were treated with the same amount of solvent and then a fungal cube was added. Experimental plates were treated with 15 µL of OPDA and then a fungal cube was added.

Table 3. Observations of each piece of moss were made after 7 days and a percentage of infection was calculated. The DE indicates the distal end was placed on top of the medium and vice versa for the PE.

PLATE		TRIAL	Percentage of infection
Experimental		1	
	DE		100
	PE		100 (CD)
Control one		1	
	DE		30
	PE		40
Control two		1	
	DE		80
	PE		100
Experimental		2	
	DE		30
	PE		100
Control one		2	
	DE		10
	PE		5
Control two		2	
	DE		100
	PE		50
Experimental		3	
	DE		100
	PE		80
Control one		3	
	DE		35
	PE		50
Control two		3	
	DE		100
	PE		100
Experimental		4	
	DE		100
	PE		100
Control one		4	
	DE		10
	PE		100
Control two		4	
	DE		100 (PD)
	PE		100

The statistical analysis showed that the control one, DE trial, p-value was 0.995 and for the control, PE trial, the p-value was 0.996. In the experimental, DE trial, the p-value was 0.982 and 0.995 for the PE trial.

Part three- Does β-glucan pretreatment protect the moss from fungal infection?

In the control plate, approximately one half of a 2 cm piece of moss was placed on the medium with the other half hanging off the edge of the divide. Next, 30 µL of β-glucan was applied to the tip on the medium. On the other side of the plate, a piece of moss was placed on the medium as previously stated and 30 µL of sterile water was placed on the tip on the medium. In the control and experimental plate, approximately one half of a 4 cm piece of moss was placed on top of the medium with the other half hanging off. After the piece was placed on the medium, 15 µL of β-glucan was applied to the piece every 0.5 cm. After 24 hours, a cube of 0.25x0.25 fungi was added to the end opposite the medium. In two trials, the DE was placed on the medium and in the other two trials, the PE was placed on the medium. On the other side of the plate, a 2 cm control piece of moss was placed across the divide, with half of it on the medium and the other half hanging off. Nothing was added to the piece. In the experimental plate, one 2 cm piece of moss was placed on the medium with half of the piece hanging off the divide and the other on top of the medium. In half of the trials, the DE was placed on the medium and in the other half, the PE was placed on the medium. After the moss was placed on the plate, 30 µL of β-glucan was added to the tip on top of the medium.

As was done in part two, daily observations were made for each piece of moss. In all cases where the fungal cube was applied, it spread over the divide and into the BCD medium.

In several of the control trials where the fungal cube was not applied, there was browning on the tip on top of the medium and no browning on the tip opposite the medium.

The mean percentage of infection for the experimental, DE trials was 100% (the piece where the fungi fell off was not counted) and 100% for the experimental, PE trials. The mean percentage of infection for the control, β-glucan trials was 12.5% and 27.5% for the control, water trials. The mean percentage of infection for the control/experimental, exp. (DE) was 50%, 100% for the exp. (PE) trials and 6.25% for the no treatment control trials.

Table 4. Observations of each piece of moss were made after 7 days and a percentage of infection was calculated. The DE indicates the distal end was placed on top of the medium and vice versa for the PE.

* indicates that the fungal cube fell off the tip of moss after day 4

PLATE	TRIAL	Percentage of infection
Experimental	1	
DE		100
PE		100
Control	1	
β-glucan		25
Water		50
Control/Experimental	1	
Exp. (DE)		0
No treatment		0

Experimental	2	
DE		100
PE		100 (CD)
Control	2	
β-glucan		20
Water		25
Control/Experimental	2	
Exp. (PE)		100 (PD)
No treatment		5
Experimental	3	
DE		25*
PE		100
Control	3	
β-glucan		15
Water		25
Control/Experimental	3	
Exp. (DE)		100
No treatment		20
Experimental	4	
DE		100
PE		100
Control	4	
β-glucan		10
Water		10
Control/Experimental	4	
Exp. (PE)		100 (CD)
No treatment		0

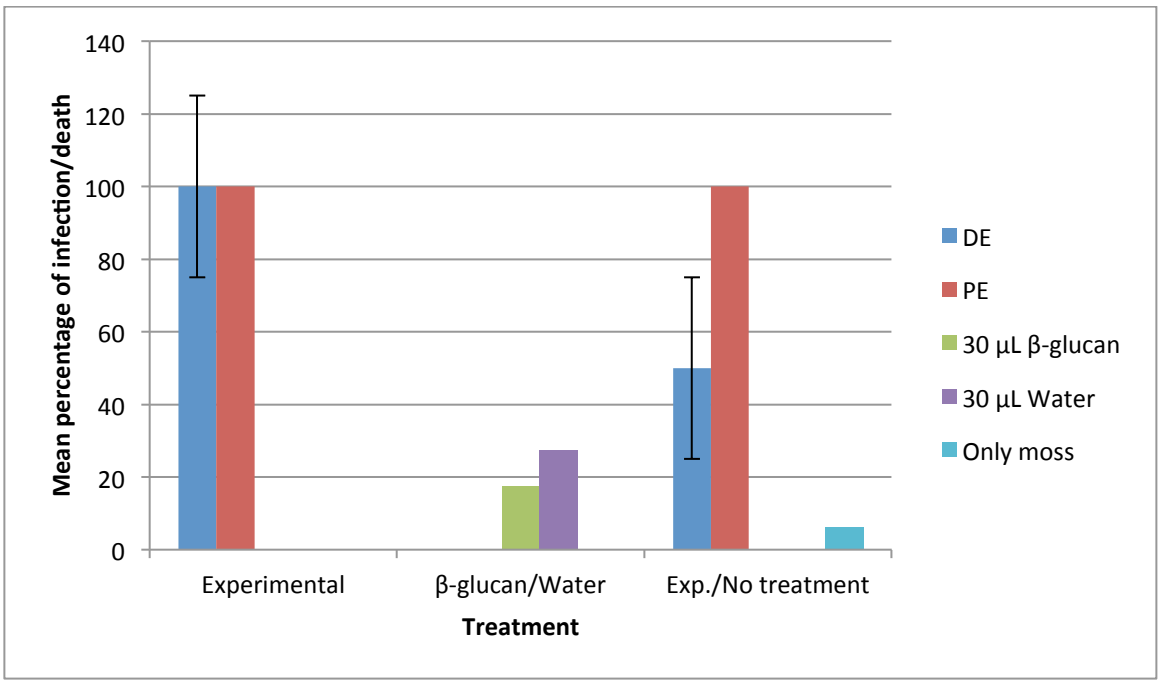

Figure 7. Infection of part three trials. The mean percentages of infection were calculated by examining the moss for appressorial injection, stem browning, chloroplast degradation and blue fungal hairs. Each piece of moss was individually observed and the percentage of the dead areas of the piece was determined. Next, the average of the dead areas of each piece was calculated and standard error bars were inserted. The experimental plate was treated with 30 µL of β-glucan and then a fungal cube was added. The β-glucan/water treatment was divided into two trials. On one side of the quarter plate, a piece of moss was treated with 30 µL of β-glucan while on the other side, the moss was treated with 30 µL of water. In the exp./no treatment trial, the piece on one side of the quarter plate was treated with 15 µL of β-glucan at every 0.5 cm; on the other side, the piece of moss was not treated with anything.

The p-value for the experimental, DE trial was 0.83 and 0.83 for the PE trial. The p-values for the β-glucan and water trials were 0.0001 and 0.0001, respectively. For the experimental portion of the control/experimental trial, the p-value for the DE trial was 0.003 and 0.83 for the PE trial.

DISCUSSION

P. irregulare causes cell death in M. cuspidatum

In the first part of the study, it was found that the fungus does infect *M. cuspidatum* cells through appressoria injection, chloroplast degradation and stem browning (Figure 4). In

all trials, the moss was infected and it was determined that *P. irregulare* was a viable pathogen for the study. The infection of *M. cuspidatum* by *P. irregulare* is consistent with other studies that examined *P. irregulare* fungal infection on the mosses, *P. patens* (Oliver, 2009) and *A. serpens* (Bowman, 2011).

Part one- OPDA does not hinder growth of P. irregulare

In each trial of this portion of the study, the fungus grew directly over the spot where the OPDA or solvent was applied without any hindrance. This indicates that the OPDA and solvent had no effect on the growth of the fungi and OPDA alone cannot kill the fungi. In each trial, the growth front appeared as an even semi-circular shape, which is further evidence that the OPDA had no effect on the growth rate of the fungi. In the majority of trials, the plate with the OPDA had the fastest growth rate. After 48 hours, all plates had been completely covered by the fungi (Tables 1 and 2).

Part two- pre-application of OPDA does not prevent fungal growth

In part two, the experimental trials showed results indicating that the OPDA trials were the same as the control two, part two trials and thus, the OPDA did not prevent or lessen fungal growth on the moss (Figure 6). This was consistent with the results of Oliver (2009), where they did a similar experiment with OPDA except they used the moss, *P. patens.* One reason why the OPDA did not prevent infection could be that the OPDA was unable to transfer into the working, defense machinery of the moss. Further research should be done to see if the OPDA is able to be absorbed by the moss and actually implemented into its defense mechanisms.

Part three- β-glucan elicitors do not prevent fungal infection

In part three, the results for the experimental trial were statistically similar to the control two, plate two trial (p-value>>0.05). It was concluded that the pre-application of the elicitor was not able to prevent or lessen the fungal growth on the moss (Figure 7). It is unknown whether the moss responded to the elicitor by activating genes that were known to combat pathogens, as was seen in Ponce de Leon *et. al* (2007). That study concluded the elicitors were able to cause cell death in *P. patens* without the application of the actual pathogen.

In the control trial with the β-glucan, the p-value was 0.0001, indicating that the trial was statistically different from the control two, part two trial and the elicitor did not harm or kill the moss. This result conflicts with that of Ponce de Leon *et. al* (2007), in that the elicitor alone is unable to cause cell death. In the water portion of the control trial, the p-value was 0.0001 which indicates that the water did not harm or kill the moss and the results were statistically different to the control two, part two moss trials.

In the experimental portion of the control/experimental trials, there were only two trials each for the DE and PE plates. Since there were so few trials, it is difficult to determine if these results provided reliable results. This must be taken into heavy consideration when interpreting the p-values. For the PE trial, the p-value>>0.05 but for the DE trial, it was 0.003. However, one of the two DE trials had a plate where none of the moss was infected while the other plate showed 100% infection. After examining the plate, the fungal cube appeared smaller than it was supposed to be and due to this, the fungus might not have been large

enough to begin infection. There is also the possibility that the β-glucan elicitor caused the cell walls to strengthen before the application of the fungi and the appressoria were not able to penetrate the cell walls. Further experiments should be done in this area in order to determine if β-glucan elicitors can prevent fungal growth in the moss.

Results involving β-glucan could have been misleading due to the amount of time between application of the elicitor and examination of the piece of moss. Previous studies applied the elicitor and then waited a maximum of 96 hours before finding the concentrations of metabolic, defense compounds. It was found that ajmalicine, a compound elicited by jasmonic acid during the SAR response, had levels that were highest after 48 hours of application (Collinge and Susarenka, 1987; Rijhwan and Shanks, 1998). Since examination was done seven days after application, the effects of the chemical compounds could have been obsolete by the time the final inspections were done.

Moss disintegration and fungal infection of BCD medium

In several trials, the moss was extremely flaccid and disintegrated upon attempts to pick it up with the forceps. On these occasions, I believe that the cell walls had degraded to such an extent that the fungus was already dead before disintegration. However, it is interesting to note that the portion of the moss on top of the medium was more likely to disintegrate than the end opposite the medium. This could have been due to the medium entering the moss cells or softening the outer epidermal layer.

In nearly every trial involving fungus, the fungi travelled across the moss and entered the BCD medium. It can be concluded with certainty that the fungi can grow on the BCD

medium as well as the PDA medium. During the qualitative analysis, it was found that the blue-stained fungal hairs were often prevalent on the end opposite the medium but tended to decrease after the divide. I believe this was due to the fungus choosing to infect the medium rather than the rest of the moss. This could have skewed some of the results where only partial infection of the moss was found. If further experiments are done in this area, a cellophane cover should be applied over top the BCD medium. This would not only alleviate the problem of the fungus traveling into the medium but it would also lessen the chance of moss disintegration.

Conclusions

One of the major conclusions of study is that OPDA or an ethanol/water solvent has no effect on the fungal growth of *P. irregulare.* Another major finding is that the fungus, *P. irregulare,* does infect and kill *M. cuspidatum* cells within 24 hours. Pre-application of OPDA is not able to prevent fungal infection or cell death in *M. cuspidatum.* The final conclusion of the experiment is that the pre-application of a β-glucan elicitor is unable to prevent fungal infection. Although the results did not yield any findings that could potentially prevent cell death in non-vascular plants, we can now move on to testing other chemicals in their ability to inhibit infection.

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