

The Impact of Endophytic *Fusarium verticillioides* on Corn Growth and Protein Composition

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ABSTRACT

Endophytic *Fusarium verticillioides* has become an emerging issue in crop and food safety. This study looks at the effects of various strains of endophytic *F. verticillioides* on the growth and protein makeup of corn (*Zea mays*). A total of 360 plants were inoculated for three treatments, with height being measured every seven days. After approximately 21 days, samples were taken from each treatment to determine if endophytic colonization occurred. Samples were also analyzed by SDS-PAGE and visualized by silver staining to evaluate any differences in the apoplastic fluid of each treatment. This study showed that there was no significant difference ($p > 0.05$) in plant height between infected and non-infected plants but there were differences noted in plant physiology and protein composition.

INTRODUCTION

This year, the United States produced almost 100 million acres of corn to satisfy global demand for food and fuel. Given the vast yield of corn, growers face a wide range of challenges, which include an emergence of pathogens that limit corn growth and production such as *Fusarium verticillioides*. Research shows that the fumonisins produced by this fungus may affect the plant host. Fumonisins (FB₁, FB₂ and FB₃) negatively affect numerous animals with life-threatening diseases such as brain damage and pulmonary edema (Yates et al., 1997). As a result, many studies investigate the relationship between corn physiology and the effects of pathogenic and endophytic *F. verticillioides* (Yates, 1997). Although some scholars conclude that the fungus impacts crop growth, few studies discuss why these varying effects occur. Therefore, this project

will inoculate corn with different strains of endophytic *F. verticillioides* in an effort to better understand their effect on corn growth and physiology.

Fungi are documented for the variety of relationships they have with plants. While some are mutualistic (e.g. mycorrhizae) or commensalistic, some fungi are pathogenic and have negative effects on plant growth and physiology, such as *F. verticillioides*. *F. verticillioides* can have a varying impact on corn plants when infected. Strains of *F. verticillioides* can either function as a plant pathogen or as an endophyte (Yates et al., 1997; Leslie and Summerell., 2006). Resulting diseases may include ear rot, stalk rot and seedling blight and it can also survive in crop debris and infect crops planted at later dates (Munkvold, 1997; Bacon, 1992). However, when existing as an endophyte, the fungus exists in a symptomless manner primarily between the cell walls and intercellular spaces (apoplast) of the stalk and leaves (Hammerschmidt, 2010; Bacon et al., 1992). Once established, fumonisin production may begin in the roots, mesocotyl and nodes and may eventually contain the highest concentrations of these toxins (Yates, 1997). Fumonisin, along with deoxynivalenol (DON) and trichothecens (T-2) are all secondary metabolites produced by *Fusarium* fungi that are classified as mycotoxins: toxic secondary metabolites formed by a fungus that are toxic to humans and animals in low doses (Woloshuk, 2012; Bennett, 2003).

Of these mycotoxins producing *Fusarium*, endophytic *F. verticillioides* and the mycotoxin fumonisin have been of significant interest since their discovery. Fumonisin FB₁, FB₂ and FB₃ were successfully isolated and characterized from *Fusarium moniliforme* (recently renamed *F. verticillioides*) strain MRC 826 in 1988 (Marasas, 1996). Two years later, a spike in leukoencephalomalacia (LEM) in horses and pulmonary edema syndrome (PES) in pigs occurred in the United States and researchers were soon able to link the consumption of fumonisin-infected corn back to the disease outbreaks by feeding horses fumonisin-infected corn (Marasas, 1996). While there are over 28 naturally occurring fumonisins, the most common and impactful is FB₁, followed by FB₂ and FB₃ (Rheeder, 2002). Fumonisin is a hydrocarbon chain with two tricarballylic acid groups and an amino terminus, which bears resemblance and structure to sphingolipids (Figure 1). Sphingolipids are lipids responsible for a range of functions including forming cell membranes, maintaining cell membrane structure and facilitating cell signaling (Marasas, 1996). When present, the fumonisins have the potential to inhibit ceramide synthase. As a result, some cell communication is compromised. In some cases, it has been documented that the resulting block of ceramide synthase activity can be used as a biomarker to indicate fumonisin toxicity. Fumonisin also have the potential to inhibit folate transport. The blocking of folate and folic acid transport can cause certain birth defects, which include exencephaly (the newborn's brain located outside of the body) and neural tube defects (NTD). While the cause of NTD or folate/folic acid's impact on preventing them isn't clear, research has been able to provide a possible link to the impact fumonisins have on these birth defects (Marasas, 2004).

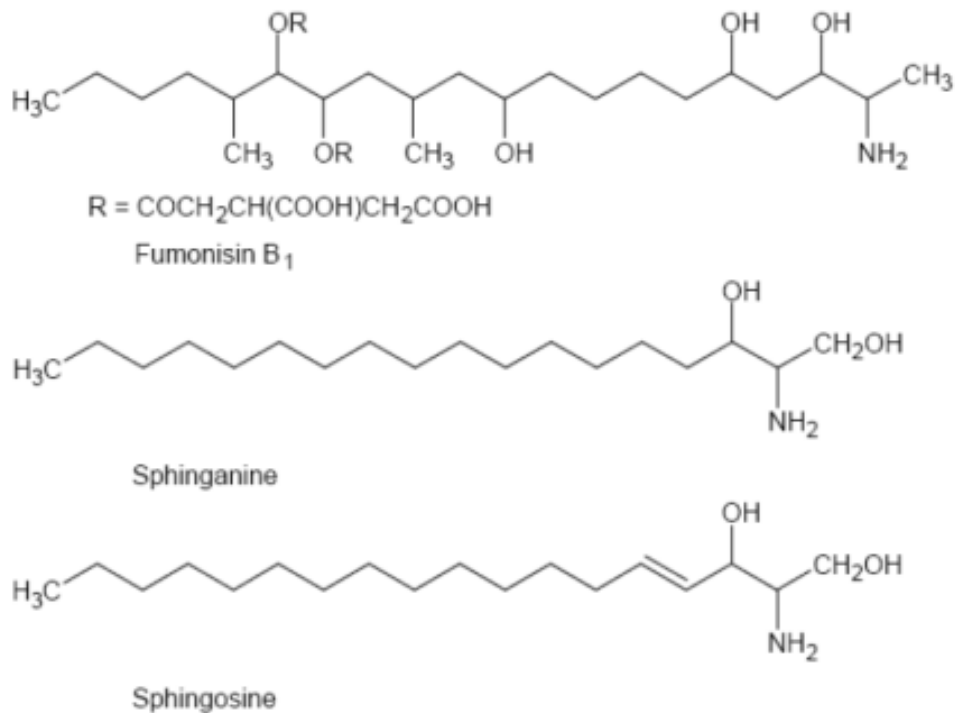


Figure 1. Fumonisin B₁, Sphinganine and Sphingosine Structure

Fusarium Toxins. Digital image. *Engormix*. N.p., 12 Aug. 2008. Web. 12 June 2013. <<http://en.engormix.com/MA-mycotoxins/articles/fumonisin-mycotoxins-increasing-importance-t1109/255-p0.htm>>.

In 2004, a study documented the effects in female mice of ingesting fumonisin-infected corn had on the development of their newborns. When the mothers ingested a corn diet containing 20 ppm FB₁ per kilogram body weight, 79% of newborn mice were diagnosed with exencephaly, while no such effects were found in the control group. When ingesting a 10 ppm diet, at least 24% of mice had observable neural tube defects. As a result of this study, the correlation between human fumonisin consumption (via homemade corn tortillas) and neural tube defects was formed (Marasas, 2004). Other studies have discovered that liver and kidney tumors became present in mice when ingesting as little as 50 ppm FB₁ in corn regularly over a two year period (Voss, 2002).

Fumonisin-contaminated animal feed was also an issue in the United States. When endophytic *F. verticillioides* and fumonisins were first characterized, corn samples were collected and tested for FB₁ in the central US from 1988 to 1995. Samples had a mean FB₁ level of 1 – 3 µg/g (ppm) with samples reaching levels as high as 5 – 38 µg/g. From 1989-1990, an LEM outbreak occurred resulting in 135 horses dying. When corn feed samples were taken, samples ranged from 1 – 126 µg/g with two-thirds of samples being above 10 µg/g. A pulmonary edema outbreak also occurred in 1989 that resulted in

1,100 swine dying in Iowa. In those fields that had documented outbreaks, FB₁ levels ranged from 3 – 330 µg/g. However in 51 fields that were classified as “non-outbreak” fields, all samples were under 10 µg/g (Desjardins, 2006). Tables 1A and 1B show the FDA Guidelines to Industry on fumonisins (FB₁ + FB₂ + FB₃ in food or animal feed (FDA, 2011).

Table 1A. Guidance for Industry Fumonisin Levels in Human Foods, According to the FDA (FDA, 2011).

Product	Total Fumonisins Allowed (FB ₁ + FB ₂ + FB ₃) (in parts per million)
Degermed dry milled corn products (e.g., flaking grits, corn grits, corn meal, corn flour with fat content of < 2.25%, dry weight basis)	2 ppm
Whole or partially degermed dry milled corn products (e.g., flaking grits, corn grits, corn meal, corn flour with fat content of ≥ 2.25 %, dry weight basis)	4 ppm
Dry milled corn bran	4 ppm
Cleaned corn intended for masa production	4 ppm
Cleaned corn intended for popcorn	3 ppm

Table 1B. Guidance for Industry Fumonisin Levels in Animal Feed, According to the FDA (FDA, 2011).

Corn and Corn By-products intended for:	Total Fumonisins Allowed (FB ₁ + FB ₂ + FB ₃) (in parts per million)
Equids and rabbits	5 ppm (no more than 20% of diet)**
Swine and catfish	20 ppm (no more than 50% of diet)**
Breeding ruminants, breeding poultry and breeding mink*	30 ppm (no more than 50% of diet)**
Ruminants ≥ 3 months old being raised for slaughter and mink being raised for pelt production	60 ppm (no more than 50% of diet)**
Poultry being raised for slaughter	100 ppm (no more than 50% of diet)**
All other species or classes of livestock and pet animals	10 ppm (no more than 50% of diet)**
*Includes lactating dairy cattle and hens laying eggs for human consumption	
**Dry weight basis	

Fumonisin toxicity issues have also occurred internationally. In 2005, research was conducted in Benin, West Africa to evaluate the fumonisin levels of silage stored corn in samples located in rural regions. Fumonisin levels in these samples ranged from 8,240 – 16,690 µg/kg. However, it should be noted that after a six-month storage period, fumonisins levels decreased significantly and fumonisins levels also dropped when the moisture content was under 19% (Fandohan, 2005). In Brazil, which is the third largest producer of corn worldwide, a study was done to look at fumonisin levels in corn samples in areas that had high rates of esophageal cancer. In the Santa Catarina region, samples tested had fumonisins levels from 2,890 µg/kg to as high as 18,740 µg/kg and it was concluded that areas of high levels of fumonisin may correlate with the high rates of esophageal cancer (van der Westhuizen, 2003). Further studies conducted by the IARC showed that fumonisin B₁ fit the parameters as a Category 2B carcinogen; that is possibly carcinogenic to humans (IARC, 1992)

While there is a good understanding of endophytic *F. verticillioides* inhabitation in plants, its impact on plant physiology is still being determined. Research conducted with endophytic *F. verticillioides* strain RRC 374 looked at the difference in the cell composition of infected and non-infected corn. Initially, there was a significant difference in the location of chloroplasts in bundle sheath cells, and height of shoots during the first 7 days after planting. However, there was no significant difference in plant height or shoot diameter between infected and non-infected corn plants at the conclusion of the experiment. There was a significant difference in root length and composition, with infected corn plants having longer roots and more secondary roots (Yates, 1997). While the results bring discussion about the specific impacts of endophytic *F. verticillioides*, the use of only one strain limits the conclusions that can be made.

While numerous studies have looked at the physiological effects of *F. verticillioides* and FB₁, many of these studies have only looked at one strain's effect. The experiment that will be conducted by this researcher will look at two different strains of *F. verticillioides*: M-1552, a weak fumonisin forming strain and M-3125, a strong fumonisin forming strain (Zitomer, 2006). Comparing these two isolates in experimental trials through an assessment of corn growth and apoplastic make-up will provide a better explanation to the behavior of certain *F. verticillioides* strains. While *F. verticillioides* has a range of hosts, corn is of the most economic importance, justifying its use in this series of experiments (Schmale III, 2013).

RESEARCH QUESTIONS

The research questions being addressed in this series of experiments include:

1. Is there a significant difference in growth when corn plants are infected by various strains of *F. verticillioides* (M-3125, M-1552)?
2. Is there a difference in the apoplastic fluid of infected plants, as compared to non-infected plants?

MATERIALS AND METHODS

Sterilization and germination protocol for corn seeds was adapted from Bacon et al (1994). Corn seeds were surface sterilized and then heat sterilized prior to planting to remove microbes. The seeds used were hybrid Silver Queen, a white sweet corn variety. Seeds were washed in 200 mL of a 5% sodium hypochlorite solution for 5 minutes then washed three times in sterile deionized water and imbibed in room temperature, sterile water for 4.5 hours. The seeds were then placed in a 60°C water bath for 5 minutes then immediately rinsed in cold water to prevent embryo damage. The seeds were then planted in autoclaved Sun Gro Redi Earth potting mix in 4" square pots. Plants were grown in a growth chamber with 16 hours of daylight at 26°C and 8 hours of darkness at 20°C. Constant 70% relative humidity was maintained throughout the experimental procedure. The plants were fertilized with approximately 14 g of All Purpose Miracle-GRO mix per 3.785 liters of water (1 tablespoon per gallon) once a week and were watered daily.

Fusarium verticillioides strains were cultured on potato dextrose agar (PDA) and incubated at 25°C for 7-14 days to allow cultures to fully develop conidia for inoculation. Once incubation was complete, 5 mL of sterile distilled water was added per plate and conidia were gently dislodged with a glass spreader. The conidial suspension was then collected and placed into a 1.5 mL tube. A 50 µL aliquot of conidia suspension was inoculated into the central whorl of the corn plant and left to incubate. Plants in the control group were inoculated with a 50 µL aliquot of sterile water. The corn plants used were growing for a week prior to inoculation. A hemocytometer was used count conidia to determine how many conidia were inoculated per plant.

ENDOPHYTIC GROWTH TEST

Plants were measured for height from the base of the stem to the top of the longest leaf. Measurements were taken either before inoculation or one day after inoculation followed by measurements taken at 7, 14 and 21 days after inoculation and averaged for each experimental group, adapted from Arias et al. (2012). A total of 360 plant measurements were analyzed for this test (3 treatments × 4 measurement dates × 10 plants per sampling date × 3 repeated trials).

ENDOPHYTIC COLONIZATION TEST

Plants were dissected 17-21 days after inoculation to obtain samples from leaves that were developing during or after the inoculation (the third and/or fourth leaf). These leaf, stem and node samples were then sterilized in a 1% sodium hypochlorite solution for one minute and then immediately washed twice in sterile deionized water. The samples were then placed on PDA and incubated at 25°C for at least 72 hours to allow growth of microorganisms from the plant tissue. During the evaluation of samples, if *Fusarium verticillioides* was observed, a sample of that plate was then cultured on another plate at 25°C for at least 72 hours to further observe microbial growth.

APOPLASTIC FLUID COLLECTION

Once the endophytic growth experiment was completed, a random sample of plants from each treatment was used to collect apoplastic fluid for analysis. The leaves from the plants used were either formed during or after the inoculation period. The leaf used to collect apoplastic fluid was washed immediately after being cut and placed in 35 mL of deionized water, 50mM sodium chloride (NaCl) or 100 mM sodium phosphate buffer (Na₂HPO₄) in a 60 mL syringe. A vacuum seal was then created with the syringe by removing all air and sealing the end with ParaFilm. The plunger of the syringe was then gently pulled back and forth for 2 – 2.5 minutes or until the leaves had a deep green color, which indicated fluid infiltration. At that point, the leaves were removed from the syringe and surface dried with paper towels. The leaf samples were then placed in 60 mL tubes with a 1.5 mL vial at the bottom to collect any apoplastic fluid and spun in a centrifuge at 2000 rpm (828 g) for 10 minutes at room temperature. The apoplastic fluid collected was then quantified and either prepped for an SDS-PAGE or stored with an equal volume of 30% glycerol at -20°C.

SDS-PAGE AND SILVER STAIN

Once the apoplastic fluid from each treatment was collected and quantified, 5 µL was used for the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Once placed in a separate vial, 5 µL of 2X Sample Buffer was added to the sample. The sample was then heated at 95°C for two minutes. The sample was then loaded onto a 12% polyacrylamide gel (Thermo Scientific, 2013), with 2 µL of PageRuler Unstained Protein Ladder (Thermo Scientific, 2013) loaded in a separate well. The gel was run at a current of approximately 150 V for 35 – 50 minutes. Once the gel electrophoresis was completed, the silver staining process began. The gel was washed twice in 50 mL of ultrapure water before staining began. Silver staining was done according to the manufacturer protocol (Thermo Scientific Pierce Silver Stain Kit, Thermo Scientific, 2013).

RESULTS

ENDOPHYTIC GROWTH TEST

At the completion of the first trial, the control group had the largest mean height at 87.9 cm, followed by the group inoculated with M-1552 at 86.04 cm, with the M-3125 group having the smallest mean height at 83.41 cm (Figure 2). However, the M-3125 group had the greatest mean height on Day 7 at 27.35 cm and the M-1552 group had the greatest mean height on Day 14 at 56.51 cm (Figure 2). At the completion of the second trial, the control group once again had the largest mean height at 120.21 cm, followed by the M-1552 group at 118.06 cm, with the M-3125 group having the smallest mean height at 115.04 cm (Figure 3). However, at Day 14, the M-3125 group had the greatest mean height at 85.81 cm (Figure 3). Observations of the two experimental groups inoculated

with the M-1552 and M-3125 strains of *F. verticillioides* included chlorosis of the leaves and high levels of anthocyanin in the stems and leaves (Figure 5,6)

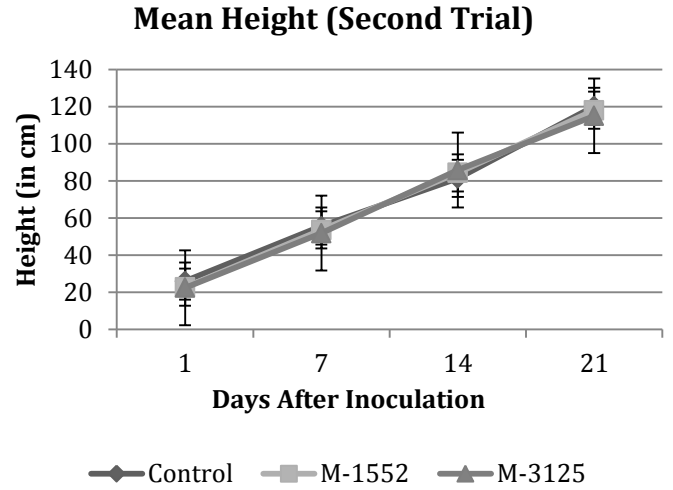
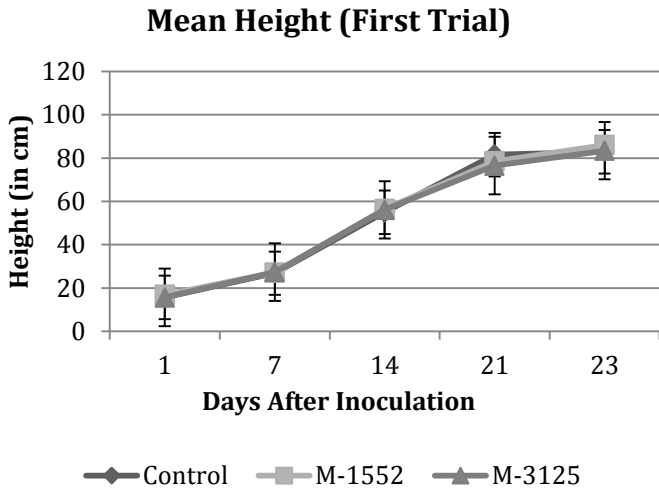
At the completion of the first and second trial of the endophytic growth test, there was no significant difference in the mean height between plants in the control group, the M-1552 group or the M-3125 group ($P > 0.05$) (Figure 4). The mean heights of each group also did not differ significantly during the 7, 14 or 21-day intervals ($P > 0.05$).

ENDOPHYTIC COLONIZATION TEST

For the first and second trial, no *F. verticillioides* was observed growing from surface sterilized tissue of water inoculated plants. In both trials plants inoculated with the M-1552 isolate had *F. verticillioides* successfully re-isolated at a rate of 40% and those inoculated with the M-3125 isolate had *F. verticillioides* successfully re-isolated at a rate of 20%. Most of the *F. verticillioides* found on plates had been from leaf samples that were still in the whorl during inoculation. There were also cases when samples from all three experimental groups were contaminated with bacteria, *Aspergillus* or *Trichoderma*.

PROTEIN COMPOSITION ANALYSIS

Protein samples from all three experimental groups in the first trial were successfully collected using all three solvents. After completing the gel electrophoresis and silver staining process, the gel was scanned (Figure 7). In the M-1552 sample collected with water, the band that was present for the control and M-3125 group at approximately 30 kDa was slightly lower than the M-1552 band found in the same area. The control sample collected with sodium chloride did not have bands at approximately 100 kDa and 45 kDa, whereas both the M-3125 and M-1552 samples had both bands. The control sample collected with sodium phosphate buffer also had a band missing at approximately 50 kDa.



Figures 2, 3. Mean height of corn plants taken during 7 day intervals after planting for each trial. The error bars at each point indicate the standard error for each sample.

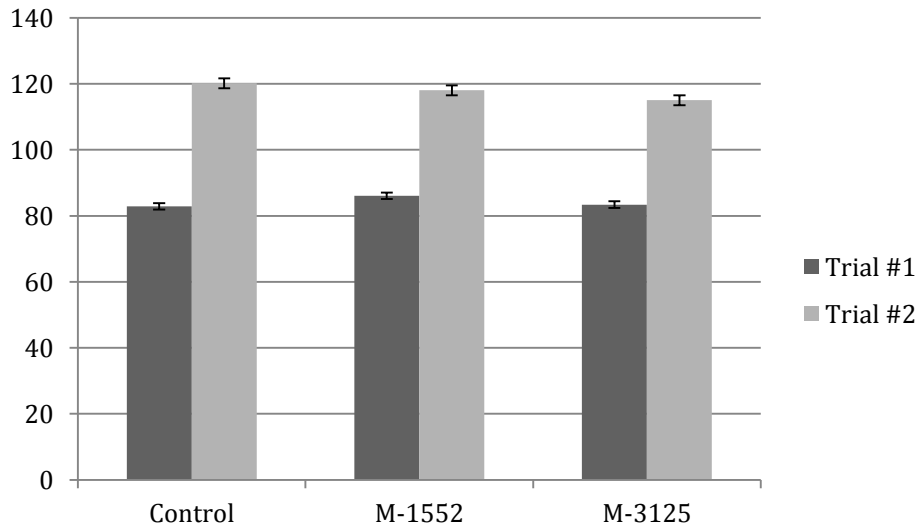


Figure 4. Mean height of each experimental group at the conclusion of data collection for each trial. The error bars on each bar indicate the standard error for each sample.



Figures 5, 6. Leaf samples collected after inoculation. Figure 5 (left) shows a sample from a plant in the control group. Figure 6 (right) shows a sample from a plant in the M-1552 group. The arrow indicates chlorosis found on the leaf, a symptom of *F. verticillioides* infection.

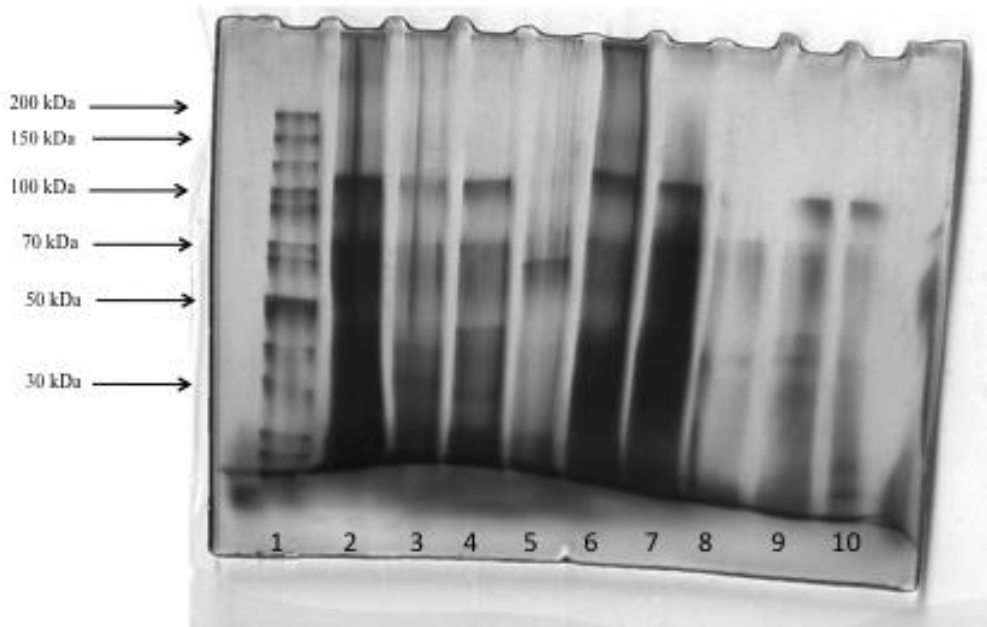


Figure 7. Silver stain gel with samples from the first trial of the endophytic growth test: Lane 1 – Molecular Weight Marker, Lane 2 – Control (Water), Lane 3 – M-3125 (Water), Lane 4 – M-1552 (Water), Lane 5 – Control (Sodium Chloride), Lane 6 – M-3125 (Sodium Chloride), Lane 7 – M-1552 (Sodium Chloride), Lane 8 – Control (Sodium Phosphate Buffer), Lane 9 – M-3125 (Sodium Phosphate Buffer), Lane 10 – M-1552 (Sodium Phosphate Buffer)

DISCUSSION

The data indicates that there is no significant difference in height between infected and non-infected plants, which correlates with the data found in the Yates study (Yates, 2004). However, physiological differences, such as chlorosis, documented in the study could become a risk in a production setting, where photosynthesis may be compromised. Future research will look to increase the sample size number, inoculate plants with a higher variety of endophytic *F. verticillioides* and increase the number of corn hybrids used.

While the hypothesis that central whorl inoculation would be successful was correct, the rate of successful re-isolation was not high. The low rate of successful re-isolation could be due to the lack of a comprehensive sampling of each plant or competition between other organisms that were competing with the *F. verticillioides* isolates. It should also be noted that *F. verticillioides* growth can be inhibited by alkaline environments and environments with high concentrations of *Trichoderma* or *Pseudomonas*, which are a noted form of biological control for *F. verticillioides* (Woloshuk, 2012; Leslie and Summerell, 2006). *Trichoderma* was found in a number of samples that were inoculated with either strain of *F. verticillioides*. Future research will look to further sterilize the potting mix used to lower the infection rate of both *Trichoderma* and bacteria while also taking more of a comprehensive sampling of plants.

While there were differences noted in the apoplastic proteins of each experimental group, the exact difference were not analyzed due to time constraints and overall quality of the protein samples. Research conducted in the Witzel study documented clearer protein bands and higher overall protein quality using the same solutions used in this study. Future research will look to use protein purification or precipitation methods to improve protein quality and quantity (Witzel, 2011). This will allow for higher quality SDS-PAGE and silver staining along with mass spectrometry to identify specific differences in apoplastic proteins between experimental groups.

The improvements in methodologies for successful isolation of apoplastic fluid will be the basis for future studies. This future research could address a number of issues that have emerged with endophytic *F. verticillioides* and corn production including unknown risks in ethanol production and phytotoxicity (Sosa, 2010) (Leslie and Summerell, 2006).

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