PROGRESS TOWARD A NOVEL MODEL SYSTEM TO INVESTIGATE FUNGAL ENDOPHYTIC SUPPRESSION OF HUMAN PATHOGENS IN SPINACH

by

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ABSTRACT

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Symbiotic microbes are known to benefit both human and plant hosts by influencing metabolic processes, immune defenses, and microbial colonization. Endophytic fungi are known to provide the host plant with benefits ranging from herbivore defense to enhanced immunity against phytopathogens. We have isolated the fungal endophytes *Stemphylium* PNW2016-02 and PNW2016-03 from commercial spinach tissue in an effort to characterize endophytic effects on plant health, including potential antimicrobial activity against the human pathogen *E. coli* O157:H7, a bacterial endophyte of spinach. Detection of *Stemphylium* in the tissue was aided by the development of a PCR-based detection method, amplifying the ITS region of the ribosomal RNA with subsequent *SnaBI* restriction enzyme digestion. Initial studies were aimed at assessing the colonization of spinach leaf tissue following *Stemphylium* inoculation onto pre-germinated spinach seeds. Following seed inoculation of our *Stemphylium* isolates, as measured by molecular detection, the fungus was shown to persist in the tissue over two weeks, at which point we observed a statistically significant enhancement of plant growth in PNW2016-02 individuals. This was surprising, as several *Stemphylium* species are known to be plant pathogens in plants including tomatoes, pears, and lentils. As previous studies demonstrated strong antimicrobial properties by PNW2016-02 *in vitro*, we hypothesize *in plantae* antimicrobial production could influence the composition of the endosphere microbial community. Future research is aimed at identifying microbial community changes during *Stemphylium* colonization in addition to *in*

plantae E. coli competition assays. With a recent rise in plant-based enterohemorrhagic *E. coli* O157:H7 outbreaks, this work has the potential to influence the development of novel plant therapeutics through the use of endophytic fungi, and therefore impacts the fields of commercial agriculture and public health as a naturally derived substitute for preventative chemical treatments.

CHAPTER 1: INTRODUCTION

Known as the endosphere, internal plant spaces hosts a vast diversity of microbes that far surpasses that of the 300,000-plus individual plant species that inhabit the Earth. Early studies of internal microbiota were limited by culture-dependent isolation procedures, however, advances in molecular and biochemical technologies, such as high-throughput sequencing, have expanded our understanding of the composition of these microbial communities, and their potential functional roles within the host. Like the internal microbiota of other species, such as humans, plant endosphere microbes can be a critical component of plant health. Understanding of specific endosphere community members and their influence on plant health can be utilized to benefit plants, just as the National Institute of Health Human Microbiome Project has enhanced our understanding of how microbiota contribute to individual well-being or disease. Genomic sequencing of hundreds of microbial strains in and upon the human body led to an explosion of research on the influences of the microbiome (Proctor et al. 2013). Continued studies into the human microbiome are demonstrating that many illnesses are associated with shifts in the structure of internal microbial communities in a manner that can favor disease (Dudek-Wicher et al. 2018; Kong et al. 2012; Dowds et al. 2015). Conversely, the human microbiome can be influenced to prevent disease and promote human health (Yousuf et al. 2019), and this phenomena is also true for vascular plants (Pérez-Jaramillo et al. 2018; Furnkranz et al. 2012). Such approaches will be critical for developing sustainable, healthy cropping systems to support agriculture under challenging scenarios of global change (Busby et al. 2017).

With advances in molecular technology, research examining questions of plant-microbe dynamics has shifted perspective, providing a closer look inside the plant endosphere to reveal

the intricacies of endophytic relationships (Hardoim et al. 2015). Microbial communities consisting of both fungal and bacterial species reside in internal plant tissue as endophytes, and these internal microbes take part in a number of interactions, from pathogenic to mutualistic. Beneficial fungal endophytes have been shown to inhibit the growth and spread of plant pathogens through competitive interactions as well as select for specific epiphytic bacteria (Arnold et al. 2007; Roberts et al. 2014). A number of endophytes produce secondary metabolites that influence microbial and plant growth as well as play key roles in competition (Strobel et al. 2002; Strobel et al. 2006; Riyaz-Ul-Hassan et al. 2012; Staniek et al. 2009). Analysis of endophyte secondary metabolites, in combination with high-throughput genomic sequencing data, holds the potential to reveal specific interactions between host plants and resident endophytes that suppress pathogens and promote plant health.

Commonly held assumptions regarding plant pathogens are also changing. For example, the fungi *Verticillium dahliae* was once recognized as an economically important pathogen of various crops; however, modern DNA-based microbial surveys of plants suggests it is a common endophyte found in healthy plants that may even suppress other fungal pathogens (Hardoim et al. 2015; Koberl et al. 2013). In addition, plant associated fungi are able to stimulate defense-related genes in their host plants when neighboring plants connected through the fungal mycelium are inoculated with pathogens (Song et al. 2010). This research suggests that exposure to particular endophytic microbes might alter not only the endosphere community, but the host community in such a way as to make plants more resistant to pathogens.

Producing safe, sustainable, and affordable food in the midst of climate change, global conflict, and a growing world population is among the major challenges of today (Urquhart et al. 2010). There is a growing recognition that knowledge of plant-microbial interactions will play an

important role in providing solutions to these challenges (Reid et al. 2012). While technological advances have allowed us to further our understanding on the diversity, ecology, and dynamics of microbial communities, we are only beginning to understand how components of these communities interact within the plant endosphere.

As complex microbial interactions are gaining increased recognition for their influence on plant species, development of model host/microbiome systems for plants was recently outlined as a top priority for plant microbiome research (Busby et al. 2017). Advancing technologies have aided in the understanding of the molecular foundations of microbiome-host physiology links, and the use of key model systems, such as gnotobiotic animals, have been essential for studying influences between a mammalian host and their residing microbiota. Similar knowledge of the relationships among plants, their microbial symbionts, and pathogens requires the development of model systems. Knowledge of plant-microbial interactions within the context of model systems holds the potential to transform agriculture, ultimately influencing our capacity to produce healthy pathogen-free plants as well as safe, sustainable food sources.

1.1 The Phytocentric Perspective

1.1.1 Diversity and Number of Plant Endophytes

The majority of plants harbor microbial symbionts, including endophytes, which range from one to several hundred species per plant (Strobel et al. 2003). It is believed that plant-microbial symbiosis has persisted since the colonization of the first terrestrial plants, and aided in the adaptation to plant survival on land (Feijen et al. 2018). Fungi mainly belonging to the group Glomeromycota are traced back to early plant life, when adaptation to land likely required improved nutrient uptake for survival (Hardoim et al. 2015). The vast amount of evolutionary time that has passed to form plant-microbe symbiosis explains their ubiquity today. Due to their

mutual presence, it is reasonable to accept that plants and their respective microbiota evolved together. This is supported by the fact that plant fungal endophytes are found in every major lineage of above-ground plants, ranging from the tropics to the arctic (Arnold et al. 2007). Each species of plant in their respective environments have developed close-interacting endophytic relationships for the most beneficial balance of survival (Arnold et al. 2007). However, despite their ubiquity, many endophytes have yet to be characterized. As more studies underline the importance of plant-endophyte interactions in plant survival, many clues to endophyte diversity are becoming apparent.

There are currently no specific estimates of the number of existing endophytes, however, approximately 90% of fungal species alone may not be described (Hyde et al. 2007), which suggest researchers are only scratching the surface of endophytic microbial diversity in general. The potential high numbers of undescribed endophytic species may, in part, be attributable to the lack of research on host plants that are viewed as irrelevant to humans, such as those not used in agriculture. However, an interest in plant endophytes due to their potential for production of natural antimicrobial compounds (Gunatilaka 2006) has stimulated endophyte research generally for all types of plants. As the tropics are among the most biologically diverse ecosystems on earth, one can assume the diverse plant species there likewise host highly diverse endophyte communities, and are ideal ecosystems to study endophytes. The use of high-throughput sequencing of the 16S rRNA gene to study bacterial endophyte communities within and around tropical rainforest plants found a strong consistency of bacterial endophyte species within plants of the same species; while among different plant species there was an increase in bacterial endophyte diversity (Haruna et al. 2017).

1.1.2 How Endophytes Benefit Plants

The plant-endophyte relationship ranges from commensal to mutualistic. The endophyte within a host plant mainly receives benefits such as nutrition, water, and physical protection; while the host plant may benefit directly from endophyte production of essential nutrients; or indirectly from production of secondary metabolites that provide protection against pathogens, insects, and herbivores (D'Amico et al. 2006). Two proposed models of endophyte mutualistic symbiosis include: constitutive mutualism, in which the endophyte is vertically transmitted through the seed; and induced mutualism, in which the endophyte is horizontally transmitted by some external source, such as the air, soil, or water (D'Amico et al. 2006). Constituent endophytes colonize the ovules of host plant seeds, persisting in successive generations of the species. Also, in areas where the same plant is cultivated for many years, endophytes remain in the soil and colonize plants through repeated horizontal transmission, developing a plant-endophyte co-evolution (D'Amico et al. 2006).

Many plants rely on endophytes to combat against disease caused by plant pathogens (Gunatilaka 2006), as a line of defense against herbivores via endophyte toxin production (Siegal et al. 1990), and in times of extreme environmental stress due to abiotic factors (Hardoim et al. 2015). Endophytes can also benefit plants by promoting growth through improved nutrient intake. For instance, the efficiency of nitrogen fixation for improved nutrient acquisition is high in endophytes such as *Gluconacetobacter diazotrophicus*, as found in sugarcane plants (Dong et al. 1994). *Gluconacetobacter diazotrophicus* was found to reside in the apoplastic fluid of sugarcane stems, which comprises approximately 3% stem volume, possibly accounting for the plant's periodic independence of nitrogen fertilizers due to this endophyte's nitrogen fixing function. Internal microbes such as arbuscular mycorrhizal fungi (AMF) can form specialized

structures in plant tissue known as arbuscules that create an interface for nutrient exchange (Hardoim et al. 2015). For example, AMF within a plant cell can exchange plant-derived carbon sources for fungal-derived nutrients such as potassium, phosphate, and nitrogen.

Recent studies examining specific host molecular pathways involved with endophyte metabolites are aimed at identifying the specific mutualistic mechanisms of plant support. Systemic resistance, or the ability of an endophyte to modulate the plant immune response in defense against pathogens, is a field of particular interest in the study of plant pathology. It is thought that due to the comparably shorter life cycle of endophytes within their longer-living host plants, they evolve faster, while selecting for beneficial characteristics that contribute to pathogen and herbivore resistance (Carroll 1988). At the initial stage of beneficial endophyte colonization, they induce plant defenses, or induced systemic resistance (ISR), that leads to higher pathogen tolerance. However, mutual endophytes eventually overcome host defense to allow successful colonization. Products of endophytic interactions provide the plant with essential compounds such as salicylic acid (SA), jasmonic acid (JA), and ethylene as signals that induce immune response (de Souza et al. 2015). These signals are received *in plantae* by pattern-recognition receptors (PAMPS) which recognize compounds present on bacterial flagella or fungal chitin (Pieterse et al. 2014). Studying the levels of ISR response to plant pathogens, and elucidating specific interactions during an infection, are crucial to understanding the roles of endophytes and how they benefit plants. It is also important to understand how manipulating these roles could potentially maximize their benefit to the plant. The development of plant-endophyte model systems could be used to further examine these roles.

Endophytes can contribute to priming, or the expression of defensive-protein coding genes, in response to exposure of a pathogen through the activation of SA-dependent and JA-

dependent pathways (Jung et al. 2012). Priming can confer an earlier and stronger immune response to a pathogen to all plant tissues, as well as neighboring plants. Generally, this heightened immune response helps defend against soil pathogens, nematodes, and chewing insects. However, with these endophytes being initial invaders themselves, they immediately begin with plant immune response manipulation to allow successful colonization.

Immune response signals may also be received when damage caused by an insect or herbivore is detected via damage-associated molecular patterns (DAMPs). Immunity is eventually developed even in the distal regions of plant tissue to protect against future exposure to the pathogen or herbivorous insect. Endophytes are central to the development of immunity, as an increase in herbivory was observed when fungal endophyte levels decreased after exogenous SA application (Bastias et al. 2017). SA, and similar compounds produced by the endophyte-symbiont host plant, regulate the pathway of endophyte-produced alkaloids, which confer herbivore resistance. One main endophyte-induced alkaloid, loline, shows specificity toward invertebrate herbivores such as aphids. This was seen when levels of endophyte-conferred resistance against aphids decreased in *Lolium multiflorum* plants after hormonal treatments (Bastias et al. 2017). Essentially, if the plant is provided with SA hormone exogenously, there is a reduction in loline alkaloid production by the endophyte, therefore leaving the plant defenseless against the aphid.

It has also been reported that herbivorous insects have a preference for individual plants based on both visual and chemical cues, which can be modulated by fungal endophytes to protect the plant from these insects (Fernandez-Conradi et al. 2017). In addition to insect preference, the same endophytes may also modify the performance of the insects after the insect has chosen a plant for foraging, decreasing the insect's efficiency to forage that plant. These benefits provided

by the endophyte are either direct, through the production of toxins harmful to the insect; or indirect, through the modification of plant organs or tissues, thus lessening the insect preference for that plant.

In addition to biotic factors that plants must withstand, many abiotic factors, such as drought, nitrogen deficiency, and salinity, exist which endophytes can provide the plant protection. These environmentally stressful conditions may be found globally where there is water depletion, flooding, and poor soil qualities. A recent meta-analysis was conducted to examine all available studies of endophytic benefits to plants under some type of environmental stress in controlled experimental conditions (Rho et al. 2017). This analysis concluded that the experimental treatment with an endophyte during environmental stress increased total biomass when compared to plants without treatment. It also suggests there is not a correlation with host-endophyte specificity, which most likely varies according to plant environments and conditions.

Certain endophytes have shown plant growth promotion, including increased root and shoot length, number of leaves, and an overall biomass (Rohini et al. 2017). 16 out of 96 endophytic bacteria isolated from the rhizome of ginger enhanced plant growth through metabolic processes such as nitrogen fixation and 1-amino cyclopropane-1-carboxylate (ACC) deaminase activity (Rohini et al. 2017). Nitrogen fixation aids in plant access to nutrients, and production of ACC deaminase inhibits plant ethylene synthesis (Rohini et al. 2017). Ethylene is a phytohormone known to induce premature leaf wilting when in high levels (Iqbal et al. 2017). The involvement of endophytes in processes such as these suggests a coevolution of endophytes and host plants to achieve optimum growth in a specific habitat.

Within some therapeutic and medicinal plants, such as *Echinacea purpurea*, it has been shown that alkamide accumulation resulting from endophyte interactions improve their

therapeutic and medicinal properties (Maggini et al. 2017). This accumulation is thought to be due to endophyte secondary metabolites, which are regulated by the increased expression of branched-chain amino acids (BCAA) decarboxylase gene. A higher expression of the decarboxylase gene was associated with endophyte-inoculated plants, resulting in the increased endophyte secondary metabolites which ultimately benefited the host plant through growth promotion and the increase in immune-modulatory and anti-inflammatory properties that this plant is known for (Maggini et al. 2017).

Endophytes can often provide the host plant with essential services, which in turn promotes the fitness of the endophyte itself. By understanding these interactions at the molecular level, there is potential for the improvement of plant life worldwide. Enhancing knowledge of how plants react in certain environments, and how their resident endophytes work to rescue them in times of stress, is especially important in areas of agricultural significance where understanding these dynamics will lead to better crop management.

1.1.3 Applications of Plant Endophytes in Agriculture

Using endophytes to promote plant growth and health, in place of using chemical agents, such as pesticides and fertilizers, holds the potential to improve the nutritional content of consumed plants and decrease damage to the fertility of crop fields (de Souza et al. 2015). By characterizing specific pathways involved in plant-endophyte interactions, currently used chemical agents in agriculture may be reduced by employing respective beneficial endophytes. Understanding and manipulating mechanisms involved in endophytic interactions may allow the improvement of almost any crop in any environment (Reid et al. 2012). While the introduction of native endophytes could improve crop sustainability, applications of specific essential endophytes could allow plants to thrive in areas they normally wouldn’t grow. In other words, in

environments that cause too much stress on the plant to thrive, such as arid deserts or flood plains, the correctly chosen endophyte may help alleviate this stress and promote growth.

Translocation of some crops to non-native areas typically results in less efficient growth levels. This is likely due to factors such as soil composition, water management, pests, and the lack of native endophytes in and around the plant. Common remedies for low plant production to address these factors include implementation of irrigation systems, traditional plant breeding, genetic engineering, and application of chemical-based pesticides and fertilizers to enhance productivity (Reid et al. 2012). Optimizing and employing essential endophytic relationships to affected crops will likely enhance production without the use of these common methods, especially those that enlist environmentally harmful chemical treatments. For instance, the agricultural wheat grass *Achnatherum inebrians* showed increased tolerance to pathogenic fungi and drought when inoculated with the endophyte *Epichloe gansuensis* (Xia et al. 2015). The results of this study suggest that endophyte-plant interactions are present in both surrounding soil and in the phyllosphere of the plant, at not only vital moments but also during the entire life cycle.

There is evidence of benefits of utilizing indigenous soils, which contain enhancing mutualistic fungi, to agricultural plants in different areas to promote growth via abiotic and biotic stress defenses (Ridout & Newcomb 2016). As previously seen, beneficial endophytes exist in the surrounding environment as well as having a well-established role in the life cycle of a plant. When an endophyte is beneficial in one region of a plant, it will also provide the same benefit in other regions of the plant (Zahn et al. 2017). Perhaps this same method may be used as a conservation measure among endangered plants, where the beneficial endophyte is derived from healthy plants and transplanted into unhealthy individuals.

Plant-parasitic nematodes are known to devastate large numbers of crops worldwide. Currently, many chemical-based nematocidal treatments of affected crops are being banned due to their negative impact on the surrounding environment. Therefore, the use of fungal endophytes, and expression of specific secondary metabolites, are being investigated as a substitute. As these endophytes are naturally occurring in many ecosystems, species known to prevent infection by nematodes and have low environmental impact is desired. A study involving the fungal endophyte *Fusarium oxysporum* strain FO162 and its secondary metabolites resulted in the identification of metabolites which served as nematocidal compounds, as well as plant growth promoters (Bogner et al. 2016). The most effective of these compounds were identified as IAA, gibepyrone D, 4-hydroxybenzoic acid (4-HBA), and methyl 2-(4-hydroxyphenyl) acetate, which showed nematocidal activity comparable to commercial chemical treatments (Bogner et al. 2016). Discovering and implementing naturally occurring compounds for plant protection or treatment of plant disease is crucial for avoiding potential negative impacts of chemical-based treatments.

1.1.4 Applications of Plant Endophytes in Industry

The biopharmaceutical industry has concentrated much effort into the discovery of bioactive phytotherapeutic compounds. Many past studies focused solely on phytochemical interactions, whereas now the focus is on endophytic associations as well (Köberl et al. 2013). With the current increase of antibiotic resistant bacterial species, there is a focus toward secondary metabolites of plant endophytes with potential antimicrobial properties. In addition, more studies are focusing on the use of plant growth-promoting endophytes to enhance the overall growth of medicinal plants.

The plant *Teucrium polium*, from which plant tissue is harvested in traditional medicine for its antimicrobial and antiseptic properties, has recently been studied in correlation to plant-endophyte relationships (Hassan 2017). Human beneficial extracts and active compounds isolated from *T. polium,* such as terpenoids and flavonoids, have been identified that have antioxidant, anticancer, antibacterial, and antifungal properties. The native habitat of the plant is arid, requiring the plant to withstand frequent environmental stress. It is hypothesized that the endophyte allow the plants to deal with these stresses. Both bacterial and fungal endophytes were isolated from *T. polium*, showing production of indole acetic acid (IAA), ammonia, and compounds capable of phosphate solubilization.

Endophyte-produced plant hormones such as IAA and phosphate solubilizers can directly benefit the plant by aiding in growth promotion and stress tolerance. Endophyte-produced IAA is namely responsible for root growth development and enhancement. Indirect growth promotion provided by endophytes includes ammonia production and degrading enzymatic molecules important for plant pathogen defense (Hassan 2017). In addition to the medicinal properties of *T.*

polium, these endophyte secondary metabolites may also contribute to growth enhancement in other commercially relevant plants such as maize (Hassan 2017).

Perhaps one of the most prominent examples of plant endophyte use in the biopharmaceutical industry is the discovery of the endophytic fungus *Taxomyces andreanae*, which was isolated from *Taxus brevifolia* that led to the production of the widely-used anticancer drug Taxol (Tanvir et al. 2017). Since the discovery of this endophytic secondary metabolite, known as taxane, other medicinally beneficial compounds have been discovered, which has led to the formation of a billion-dollar industry (Tanvir et al. 2017).

With the use of plant-beneficial endophytes, there exists a potential of large-scale plant conservation by providing mutualistic symbionts that are not native to the plant's microbiome. As seen as a benefit to the human digestive tract, for instance, microbiota that colonize the gut co-exist as a mutual relationship, providing services such as defense against pathogenic microbes and immunity strengthening. Natural killer T (NKT) cells of the human innate and adaptive immune system that interact with commensal microbes of the intestinal mucosa to provide homeostasis and prevention of inflammation (Dowds et al. 2015). Even in times of severe intestinal bacterial infection, such as with *C. difficile*, fecal transplants containing a beneficial microbe can be used as a treatment measure to control pathogen levels through direct competition. This same concept, introducing foliar endophytes isolated from healthy plants into severely diseased plants for treatment, has been studied previously (Karlsson et al. 2014).

The endangered, fungicide-dependent plant *Phyllostegia kaalaensis* requires controlled greenhouse growth conditions since its extirpation from the wild. Without monitoring, the plant will quickly parish in the wild due to the frequent fungicidal treatments also killing-off beneficial microbes. Foliar fungicides used for these treatments contain active ingredients such as

azoxystrobin and bixafen, and which target known fungal pathogens, but may also be very broad, leading to a negative impact on surrounding plants, soil, and overall microbial community (Karlsson et al. 2014). With broad-range fungicidal treatments leading to unnecessary removal of surrounding beneficial microbes, whether in the soil or other plants, there is a need to examine alternate treatment regimes. Diseased and defenseless *P. kaalaensis* has been shown to recover following the transmission of beneficial microbiota from healthy, wild-grown relatives (Zahn et al. 2017). The once greenhouse and fungicide-dependent plant is now able to survive in natural conditions with these inoculation treatments.

As more plant species become negatively impacted by climate change, deforestation, and increasing human population, there will be a stronger need for large-scale restorations. Therefore, the use of endophytic treatment in failing plant populations may have the potential to increase successful restoration, and this is especially important for those plants that have agricultural relevance.

1.2 Endophytic Bacteria

1.2.1 The Function of Bacterial Endophytes

To date, studies of bacterial endophytes that have been isolated from both cultivated and non-cultivated plants suggest that the most frequently represented phyla include *Proteobacteria, Firmicutes, Actinobacteria,* and *Bacteriodetes* (Haruna et al. 2017). In addition to these, *Cyanobacteria, Acidobacteria,* and *Chloroflexi*, are isolated at a lower occurrence. Some of the commonly isolated genera belonging to these phyla include *Bacillus, Streptomyces, Pseudomonas,* and *Lysinibacillus*. The majority of bacterial endophyte studies are focused toward the discovery of active microbial compounds that may beneficially contribute to human health. However, while work in antibiotic discovery via endophyte research has been covered

extensively, the beneficial effects that these endophytes have on their host plants may be often overlooked, or at least understudied.

The rhizosphere of a plant can contain 10^{10} bacterial cells per one gram of soil, and within this, 10,000 various bacterial species (Reid et al. 2012). Among the diverse bacterial community, many species occupy niches on and within the aboveground parts of the plant, or the phyllosphere. As with all endophytes, endophytic bacteria can be horizontally acquired from the environment each generation by remaining in the soil and attaching to seeds. The endophyte becomes internalized once the seedling emerges. Vertically transmitted endophytic bacteria maintain colonization in successive generations of a host plant by remaining in the embryo of seeds. With either mechanism of transmission, beneficial endophytes are likely selected for when they are advantageous to plant survival and growth.

1.2.2 Beneficial Endophytic Bacteria

There are numerous descriptive examples of bacterial endophytes benefiting host plants by diverse mechanisms (Eljounaidi et al. 2016; Ma et al. 2016; Tian et al. 2017; Hong et al. 2016). In addition to promoting plant growth through improved nutrient intake and stability against abiotic factors, endophytic bacteria can improve plant productivity and stress tolerance in the absence of pesticides and fertilizers, as well as facilitate phytoremediation of heavy metals and hydrocarbons (Busby et al. 2017). Endophytic bacteria can also prevent colonization of plant pathogens that lack the epiphytic stage necessary for proper chemical treatment, meaning applied treatments do not efficiently reach the internalized pathogen. Currently, insecticides are used to prevent colonization of insect-transmitted plant pathogens, but the use of endophytes as biocontrol agents is a promising field of study.

The bacterial plant-pathogen *Candidatus* affects grapevine growth, among other plant species, and has shown sensitivity toward *Dyella*-like bacterial endophytes in young plants (Lidor et al. 2017). Using an anti-pathogen endophyte such as *Dyella*-like bacteria as a biocontrol agent may improve the efficiency of maintaining valued crops as opposed to using ineffective chemical treatments. Another study involving the effects of beneficial bacterial endophytes on grapevine growth includes the inhibition of *Botrytis cinerea* mold by the bacterium *Burkholderia phytofirmans.* Grapevine plants challenged with *B. cinerea* were studied to elucidate mechanisms of endophyte defense after *B. phytofirmans* PsJN treatment (Miotto-Vilanova et al. 2016). These results suggest that in addition to a direct antifungal activity by the endophyte, there was also priming of defensive mechanisms via H_2O_2 accumulation (i.e. reactive oxygen species, ROS), enhanced expression of defensive genes, and modulation of carbohydrate metabolism. These activities were not observed in non-infected plants, and ultimately promoted growth and survival (Miotto-Vilanova et al. 2016).

1.2.3 Human Pathogens as Endophytes

With an increase in the production of fresh and/or minimally-processed produce, such as lettuce, spinach, etc., there has been a related increase in foodborne illnesses caused by bacteria, viruses, and protozoa in the phyllosphere and rhizosphere of agricultural plants (Whipps et al. 2008). Some pathogenic bacteria, such as *Listeria* spp., occur naturally in surrounding soils and may spread to plants directly, or adhere to plants via water splash (Whipps et al. 2008). However, some bacterial endophytes, such as *Clostridia* spp., can exist as pathogenic microorganisms within the endosphere of a plant (Whipps et al. 2008). *Escherichia coli* O157:H7 is known to be a human pathogen found on the surface of plants, such as those that

have caused previous outbreaks, but it can also exist as a plant endophyte, making typical surface-sterilization measures ineffective against it (Shaw et al. 2008).

In addition to the use of manure fertilizer and contaminated irrigation sources, it seems that post-harvest handling and processing may also be a route of contamination of human pathogens, internalizing in the plant tissue after exposure (Brandl 2008). To help rid agricultural produce of internal pathogen colonization, more research needs to examine potential measures of *in plantae* pathogen inhibition and elimination via natural competitions of endophytic organisms.

1.3 Endophytic Fungi

1.3.1 The Function of Fungal Endophytes

As mentioned, fungal endophytes are long-time residents of terrestrial plants, having coevolved with them since their existence. Every plant that has been observed and sampled contains at least one species of endophytic fungi, found as a vast network of closely-associating microbes in most plant tissues with many functions still unknown (Reid et al. 2012). Plants and fungi have co-evolved through mutualistic interactions, providing one another adaptive benefits for optimal survival. In addition to those found in roots, many fungal endophytes reside within the above-ground plant tissue to help protect against both biotic and abiotic factors.

There is genetic linkage between arbuscular mycorrhizal fungi (AMF) and root nodule symbionts, which is evident of plant-endophyte evolution (Hardoim et al. 2015). AMF are members of the class *Glomeromycetes*, which are the most abundant endophytes (39%) in land plants, and are therefore of ecological and economical importance due to their ubiquity and obligatory lifestyle among agricultural plants (Hardoim et al. 2015). For example, AMF associate with the roots of 80% of land plants, extending roots by the formation of hyphae, and aid in the acquisition of nutrients, minerals, and water for the plant host (Reid & Greene 2012).

The fungal endophyte-plant relationship is perhaps the most important factor to consider when examining the success of today's evolved plant life.

1.3.2 Beneficial Endophytic Fungi

Fungal endophytes provide defense against plant pathogens and herbivores by several mechanisms, including through the production of chemical compounds such as alkaloids (Reid & Greene 2012). The fungal endophyte *Clavicipitaceae* are found to form intercellular communication networks among neighboring grass plants to produce defensive toxins against insects and grazing animals (Reid & Greene 2012). The fungal endophyte *Neotyphodium* has been found to benefit perennial ryegrass and fescue by providing protection against invading weeds and phytopathogens (Devi et al. 2015). Resident fungal endophytes also contribute to host plant protection via compounds that deter or inhibit threats, similar to mammalian immune responses mounting defensive compound cascades to recognize and neutralize foreign invaders to the system.

Other benefits provided to a host plant by endophytic fungi include improved nutrient uptake and processing, which may result in increased biomass via stimulation of plant growth (Devi et al. 2015). The introduction of the endophytic fungus *Cladorrhinum foecundissimum* showed improved phosphorus uptake via nitrogen transfer along with increased plant height in cotton (Devi et al. 2015). Fungal endophytes can also protect against abiotic stress factors, such as heat, drought, high salinity, and presence of heavy metals (Reid & Greene 2012). It is becoming more apparent that the survival of plant life in these types of extreme environments is only because of their developed relationships with beneficial endophytic fungi. Examining the specific interactions involved in unique defense mechanisms of plants can better our understanding of natural plant protection.

Many endophytic fungi will only produce secondary metabolites while colonized within the host, and are not produced when the endophyte is cultured outside of the plant (Souvik et al. 2012). Many of these metabolites, including alkaloids, polyketides, and terpenoids, have been identified and characterized as having antibacterial properties. Other fungal metabolites are known to have therapeutic properties, such as antineoplastic paclitaxel, camptothecin, anticancer compounds podophyllotoxin and deoxypodophyllotoxin, the antidepressant hypericin, and the insecticides azadirachtin A and B (Souvik et al. 2012).

1.4 Stemphylium and Endophytic relationship

1.4.1 Stemphylium as a Model Organism

Stemphylium is a filamentous fungus belonging to the phylum Ascomycota, in the order Pleosporales (Saha et al. 2014). Estimated numbers of existing *Stemphylium* species vary, ranging from 20 to 30 (Câmara et al. 2002 ; Kirk et al. 2001), and even as high as 150 (Wang & Zhang 2006). In the genus, morphological characteristics, such as conidia length and appearance, have been traditionally used for species delineation (Woudenberg et al. 2017), however, molecular studies using, for example, ITS sequencing are expanding our understanding of the genus. A recent revision of *Stemphylium* species by Woudenberg et al. (2017) distinguished 28 species-clades, with many current species names being synonymous, and an additional five novel species being recognized using molecular, rather than morphological data alone. Another study examining the chemical structure of *Stemphylium* secondary metabolites, also suggest that certain taxa in the genus, namely *S. alfalfa* and *S. herbarum* with *S. vesicarium*, should be synonymized (Olsen et al. 2018).

While *Stemphylium* species are known to enter a mutualistic relationship with host plants, some species are commonly recognized agents of disease, including purple spot disease in

asparagus and brown spot disease in pears (Graf et al. 2015). Further, *S. globuliferum* has been implicated in yellow leaf spot disease in sugar beets, as well as being identified as a plant pathogen in legumes, clover, and alfalfa (Hanse et al. 2015). Other *Stemphylium*-associated plant disease affect onion, garlic, parsley, and tomato (Olsen et al. 2018).

The diversity of biological processes that some fungi possess can vary greatly among hosts, and the same may be true for *Stemphylium* species. Functional roles can be reversed between pathogenicity and mutualism depending on the plant species in which the endophyte resides (D'Amico et al. 2006). The balance between environmental stress and primary plant defenses appears to dictate the functional role of the fungus (D'Amico et al. 2006). Additionally, the timing of the shift from being a beneficial endophyte to a pathogen may be an important factor in allowing plants to mature before the onset of disease (D'Amico et al. 2006). The development of a mutual symbiotic relationship depends on the species of both the plant and the potential endophyte, in addition to conditions that favor the relationship.

To better comprehend the dynamics of plant-endophyte interactions, there is a need to develop model systems. Fully understanding how agriculturally-important plants withstand both biotic and abiotic stress through the use of endophyte treatment may have a great impact on food production sustainability (Busby et al. 2017). The fungal endophyte, *Stemphylium*, is a desirable candidate for use in such a model due to its flexibility on the plant-pathogen-to-plant-endophyte spectrum, in addition to the many desirable compounds, including antibiotics, which are produced by members of the genus.

1.4.2 Endophytic interactions by Stemphylium species

Stemphylium species may also indirectly contribute to plant health through the by-products of plant-endophyte interactions. The results of a study involving the yellow serradella pasture plant (*Ornithopus compressus)* inoculated with *Stemphylium* showed an increase in total nutritive value for foraging (Santamaria et al. 2017). This increase in nutrition seems to be facilitated by the by-products of *Stemphylium* interactions with the plant, as measured by increases in available crude proteins and essential minerals, and a decrease in the levels of toxic elements. For instance, resulting plants showed an increase in essential minerals such as boron, phosphorus, and sulfur; as well as a decrease in phytotoxic elements such as aluminum and lead (Santamaria et al. 2017).

1.4.3 Metabolites of Stemphylium

As seen above, secondary metabolites of endophytic fungi can confer advantages to the plants they colonize by either direct or indirect interactions with phytopathogens and herbivorous insects and animals. In particular, *Stemphylium* spp. have been shown to induce antibacterial properties via secretion of secondary metabolites such as stemphol, including stemphol A and stemphol B. When compared to the broad-spectrum antibiotic ciprofloxacin, certain common pathogenic bacteria, such as *E. coli, B. cereus,* and *S. aureus*, showed similar sensitivity to the stemphol compounds, with added potency toward *E. col* (Zhou et al. 2014). Chemical analysis of *S. globuliferum* found secondary metabolites alterporriol, altersolanol, stemphypyrone, 6-O-methylalaternin, and macrosporin, all with bioactive properties (Debbab et al. 2008). In our lab, *Stemphylium*-like plesporelean endophytes isolated from commercial spinach have shown inhibitive properties toward *E. coli* O157:H7 following *in vitro* treatments.

1.5 Future Directions for Endophytic Research

Continued research in the field of endophyte-plant associations is a priority due to the potential outcomes that could benefit economically important crops. Not only the successful production of these plants is important, but also related impactful issues such as consumer health and safety, bioremediation, environmental preservation, and food sustainability, all may be improved by implementing the right endophyte treatment. Use of endophytic treatment in agriculture and bioremediation as opposed to chemical treatment, for instance, may not only improve plant health, but also replace environmentally harmful runoff. Also, with an increasing human population and decreasing food supply and sustainability, endophytic treatment could help encourage the growth of plants in less hospitable environments, such as regions with vast drought or flooding. Just as the human microbiota influences our disease-susceptibility, pathogen resistance, metabolism, and many other aspects of health, the endosphere of plants contain similar interactions that promote plant health.

To better study the integrant involvement in plant-endophyte relationships, it is crucial to develop new model systems to examine the intricacies involved. One possible endophyte to focus on may be *Stemphylium* due to its similarities to *Alternaria*, and its abundance in agriculture. There also exists a need for increased awareness for research funding, specifically research involving high throughput sequencing of microbial communities and the environmental changes that influence them. Another important step forward would be the isolation of antimicrobial compounds discovered as endophyte products that may be natural sources of novel antibiotics. With antibiotic resistance on the rise, the development of effective antibiotics is very crucial. Lastly, there is importance in research based in endophyte transfer for the purpose of soil remediation in deficient crop fields. In fields that have been either over-farmed and depleted of

nutrients, or polluted in some way, the transfer of beneficial endophytes to the soil or the plants themselves may help. There has been success in the transfer of mycorrhizal soil, where plants have recuperated after exposure to endophytic-enriched substrate. However, more work has to be done to identify which endophytes can directly benefit a plant's phyllosphere, not just the below-ground rhizosphere.

1.6 Description of Project Goals

The work shown here addresses the initial steps necessary to develop a plant-endophyte model system. It involves two strains of *Stemphylium*-like fungal endophytes that were isolated in our lab from commercial spinach, named PNW2016-02 and PNW2016-03. As described above, these endophytes displayed *in vitro* inhibition of human pathogen *E. coli* O157:H7. To begin examining the possibility of this antimicrobial property *in vivo*, the following goals were pursued:

1. High-throughput spinach seed inoculation with PNW2016-02 and PNW2016-03 endophytes using a novel 96-well plate seed germination system.
2. Automated seedling growth of both treated and non-treated plants.
3. Molecular detection assay for determining successful inoculations using our designed pleosporalean-specific ITS-1 primers.
4. Distinction of internalized *Stemphylium* presence among other closely related pleosporalean endophytes, such as *Alternaria*, using the restriction endonuclease *SnaBI*.

The project described here was successful in meeting these goals. Therefore, with the use of this system, work can begin to examine whether or not the *in vitro* antimicrobial ability of these endophytes can also be seen *in plantae*.

CHAPTER 2: MATERIALS AND METHODS

2.1 Preparation of Stock Fungal Suspension

The fungal endophyte *Stemphylium* strains PNW2016-02 and PNW2016-03 were isolated from commercially available spinach plants (*Spinacia oleracea*) and grown on potato dextrose agar (PDA). Actively-growing mycelium of the two strains were removed the surface of the PDA and placed in 500 mL flasks containing 150 mL sterilized dH_20. After four weeks of growth in flasks, spore-containing fungal mycelia were visible. The fungal mycelium was removed from the flask and placed into sterile 50 mL conical tubes with 25 mL of water from the flask. A Tissue-Tearer surface-sterilized with 95% EtOH was used to homogenized the fungal tissue until a uniform liquid consistency was achieved. The resultant stock fungal suspension was then used to inoculate spinach seeds. Additionally, 1.0 mL of the fungal suspension was removed and serially diluted to a $1:10^{-6}$ dilution for plating on PDA to determine the approximate cell concentration of stock fungal suspension.

2.2 Seed Inoculation and Initial Germination

Ferry-Morse brand Matador spinach seeds were purchased from a local retailer. Pre-packaged, sterile, non-tissue treated 96-well plates were used as germination containers for the spinach seeds. Vermiculite was ground to a fine powder, autoclave sterilized, and placed into each plate well as a germination substrate. Seeds were surface sterilized with 10% sodium hypochlorite (NaOCl). One seed was then placed into each of the plate wells, which were then inoculated with sterile pre-packaged disposable pipets using 0.5 ml of stock fungal suspension for each of the two endophyte strains, PNW2016-02 and PNW2016-03, being tested in separate

germination experimental treatments. A control plate was also prepared in a similar manner; however, each well in this plate was 'inoculated' with sterile dH_20 rather than the stock fungal suspension. After inoculation, additional sterile vermiculite was used to completely cover each seed in the wells. Plate lids were put into place to completely enclose the prepared wells, and then each of the three plates were placed in a growth chamber for a 7-day germination period under constant soft-light from iridescent GE light bulbs (GE Lighting, Peru).

2.3 Preparation of Plant Containers

Plant containers were prepared from aluminum 8 oz. beverage cans that were thoroughly cleaned with 10% bleach, and then cut in half. The cut bottoms of the containers were taped with colored masking tape for safety and to designate each of the treatment and control plants, using one color for each of these, in the growth chamber. Jiffy brand peat soil disks were placed into each container as a growth medium. Each of the prepared containers were then placed on a tray and covered with aluminum foil for sterilization in the autoclave for 20 min. in the Liquid 4 cycle.

2.4 Final Inoculation Experiment Set Up

Seedlings from each of the 96-well plates were aseptically transplanted to plant containers that were pre-moistened with sterile water. Each of three types of color-coded containers representing the two experimental treatments and control plants were placed in a sterilized plastic plant starter trays. A total of 18 containers were prepared for each experimental and control treatments, and these were placed in three trays (3 rows of 6 plant containers in each tray) for a total of 54 plants in a random block design to mitigate potential differences in growth conditions, such as light exposure, within the growth chamber. Plant placement in the random

block design was determined by first assigning a number to each row and column across the three trays to accommodate all plants. Random numbers across the range, 1–54, were generated for each container for placement in the trays according to the corresponding numbered position in one of the trays.

2.5 Growth Chamber and Conditions

All trays were placed in a growth chamber, which was consisted of a sterilized shelves, over which was suspended an iridescent lighting unit containing the GE bulbs mentioned above, that were wrapped in 6 mil plastic sheeting to minimize fungal contamination from sources outside of the chamber. Plants were watered with a mixture of sterile water and Hoagland's No. 2 Basal Salt medium (Sigma-Aldrich Co., MO, USA) prepared at a ratio of 6.05 g of Hoagland's medium per gallon of water. This liquid growth solution was supplied to the plants from a sterile five gallon bucket furnished with a pump with an automated timing system that delivered water to the trays. The growth solution was pumped into 2 cm deep groves at the bottom of each tray, and the automated delivery kept a constant supply of the growth solution in the groves throughout the course of the experiment. The growth solution was provide to the plant from the tray groves via a sterilized paper wick that was placed under the disk and folded over the container rim to reach the grove at the bottom of the tray containing the solution. Plants wicked growth solution from the tray groves as needed. Lighting was provide to the plant in the chamber for a period 12h on/ 12h off, controlled by an automated timing system. The experiment was run for two weeks. At the end of the experiment each plant was removed from the containers, rinsed with sterile water to remove debris, photographed, measured from the base of the stem to the tip of the longest cotyledon, and then placed into labeled cryogen tubes. Plant tissue was stored at -

80°C and later removed for DNA extraction required for the molecular fungal endophyte detection protocol.

2.6 Molecular Endophyte Detection

2.6.1 Specific Primers and Optimization

A method for detecting the *Stemphylium* strains (Pleosporales, Ascomycota) in plant tissues was develop using a combination of polymerase chain reaction (PCR) amplification followed by a restriction enzyme assay to confirm successful endophyte inoculation of the plants. The PCR amplification phase of the molecular detection protocol first targets fungi in the Pleosporales, an order that in addition to *Stemphylium* includes the common and widespread endophyte *Alternaria*, while the restriction enzyme assay phase distinguishes between species in *Stemphylium* versus other genera, such as *Alternaria*. We designed Pleosporales-specific primers that target the internal transcribed spacer (ITS) region of the nuclear rRNA gene which had the following sequences: Forward – 5'- CAC CAG GAC CMA ACC ATA AAC -3'; Reverse – 5'- GCA AAG CTT GAG GGT ACA AAT G -3'. Primers were ordered from Integrated DNA Technology (IA, USA). 100µM primer stocks were prepared and placed at -20°C, from which 10µM working aliquots were prepared for each assay.

To determine optimal PCR conditions for use of with our Pleosporales-specific primers, tested various annealing temperatures in a touchdown PCR using genomic DNA extracted from mycelium of our previously isolated *Stemphylium* endophyte strains, PNW2016-02 and PNW2016-03, as well as PNW2016-04 (*Chaetomium* spp.) and a commercially purchased strain of *Alternaria*. Touchdown PCR used preset thermal gradient conditions for annealing temperature tests in a Mastercycler Nexus thermocycler (Eppendorf North America, NY, USA). The following cycle conditions were determined to be optimal, and were then preset into the

thermocycler for all future PCR assays: 95°C (4:00 min.), 95°C (1:00 min.), 52°C (0:45 min.), 72°C (1:30 min.), and 72° C (4:00 min.). Amplification success of fungal ITS nrRNA gene amplicons was confirmed by gel electrophoresis in a 1% agarose gel containing ethidium bromide (EtBr) in Tris-acetate EDTA (TAE) running buffer. Gels were run at 80V/100mAmp for ~30 minutes.

2.6.2 DNA Extraction and PCR

All plant material was ground and pulverized using liquid nitrogen in separate sterile mortars and pestles, and then placed into labeled tubes. All DNAs were stored at 20° C until use in the PCR phase for endophyte detection. DNA extraction was performed on all samples using a DNEasy Plant Mini Kit (Qiagen, KY, USA), following the kit protocol with the addition of an initial heating step at 65°C for 10 min. Isolated genomic DNA from each sample was then measured for quality and concentration using a NanoDrop 2000 (Thermo Scientific, IL, USA) prior to use in PCR.

For PCR, a standard template concentration (C_f) of 2.5 ng genomic DNA/µL was used, which was calculated using the following formula where initial volume (V_i) of each sample was determined for 25 µL reactions:

$$V_i = (C_f \times V_f)/C_i$$

PCR reactions were carried out on all samples using the predetermined optimized PCR cycling conditions (see above), and confirmed in gel electrophoresis following the protocol outlined above.

2.6.3 Restriction Endonuclease Digest Assays

The restriction enzyme assay phase used the *SnaBI* restriction endonuclease (New England BioLabs, MA, USA), which cut pleosporalean fungal amplicons generated in PCR to distinguish those from within the genus *Stemphylium* versus those representing other species from the Pleosporales. All amplicons were assayed using the following reaction volumes: 5.0 uL DNA, 0.5 uL *SnaBI*, 1.0 uL 10x enzyme buffer, and 3.5 uL dH20; for a total reaction volume of 10.0 uL. The reactions took place at 37°C for 1 hour, and the resultant assays were confirmed in gel electrophoresis following the protocol outlined above.

CHAPTER 3: RESULTS

3.1 Initial Spinach Seed Germination

Our novel method developed to inoculation spinach plants with fungal endophyte strains, began with germination of the spinach seeds soaked in our stock solution of the *Stemphylium* endophytes, either PNW2016-02 or PNW2016-03. In this initial phase of the inoculation experiment, we had successful germination of spinach seeds, with the germination rates for each set of inoculations and the control shown in Table 1. An image of the 96-well plate germination container can be seen in Figure 1 with seedlings presumably having internalized PNW2016-02. Germination success rates were generally low for all seeds included in the germination containers, with all treatments producing rates below 50%. Overall, germination success rates were higher for endophyte strain inoculated seeds, with seeds from the PNW2016-02 treatment having rates of germination that were nearly double those of the other treatments. For the final phase of the inoculation experiment, we selected 18 of germinated spinach seeds from each treatment to test if the inoculated endophyte strains would persist in the spinach plants as they grew into seedlings.

Table 1: Rates of successful spinach seed germination in the initial phase of our experiment after inoculation with fungal endophyte strains or sterile dH_20 in the 96-well plates.

Treatment	**Germination Rate**
PNW2016-02	39/96 = 41%
PNW2016-03	28/96 = 29%
Negative Control (dH_20)	21/96 = 22%

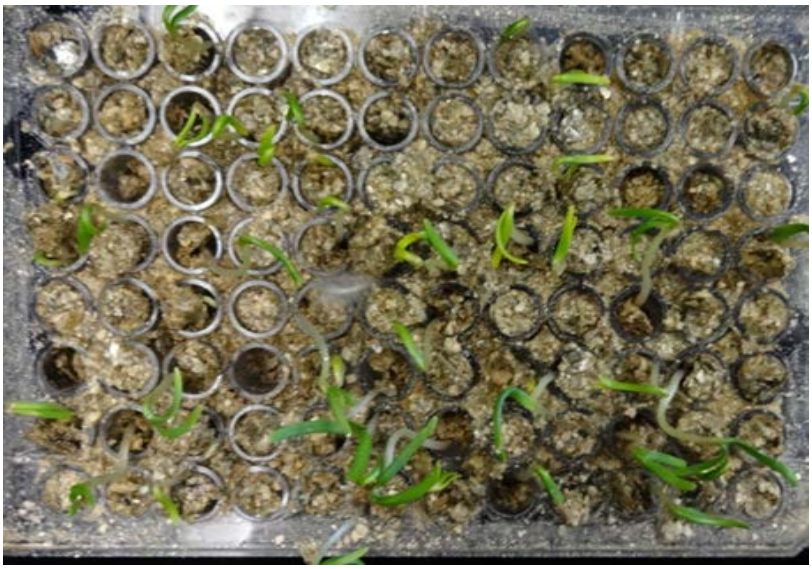

Figure 1: In the initial phase of the inoculation experiment an image of the 96-well plate germination container shows successfully germinated spinach seeds after inoculation with fungal endophyte strain PNW2016-02. Vermiculite was used as an absorbent substrate to retain the applied fungal spore suspension for the duration of the germination period.

3.2 Height Measurements of Plants from the Experimental Treatments

In the final phase of the inoculation experiment following a 14-day growth period, spinach plants moved into the growth chamber were measured for height at the end of the experiment. The average height for plant from the PNW2016-02 inoculation treatment was 9.63 cm; with the tallest being 15.4 cm, and the shortest being 5.9 cm. For the PNW2016-03 inoculation treatment average height was 6.88 cm; and ranging from 5.1 cm to 7.9 cm. The average height of the control plants was 5.1 cm; and ranging from 4.1 cm to 6.4 cm. Plants from the inoculated treatments appeared to be generally more robust than non-treated plants, with those of the PNW2016-02 inoculation treatment being most notably so (Fig. 2). This trend was also reflected in the plant height measurements, with plants from both experimental treatments being statistically taller than the control plants ($p < 0.001$ for both treatments), and the

PNW2016-02 inoculation treatment plants also showing a statistically significant height increase ($p < 0.01$) compared to those of the PNW2016-03 inoculation treatment (Fig. 3)

Figure 2: Images of two-week old spinach seedlings with greatest height after inoculation with A) PNW2016-02; B) PNW2016-03; C) un-inoculated control plants.

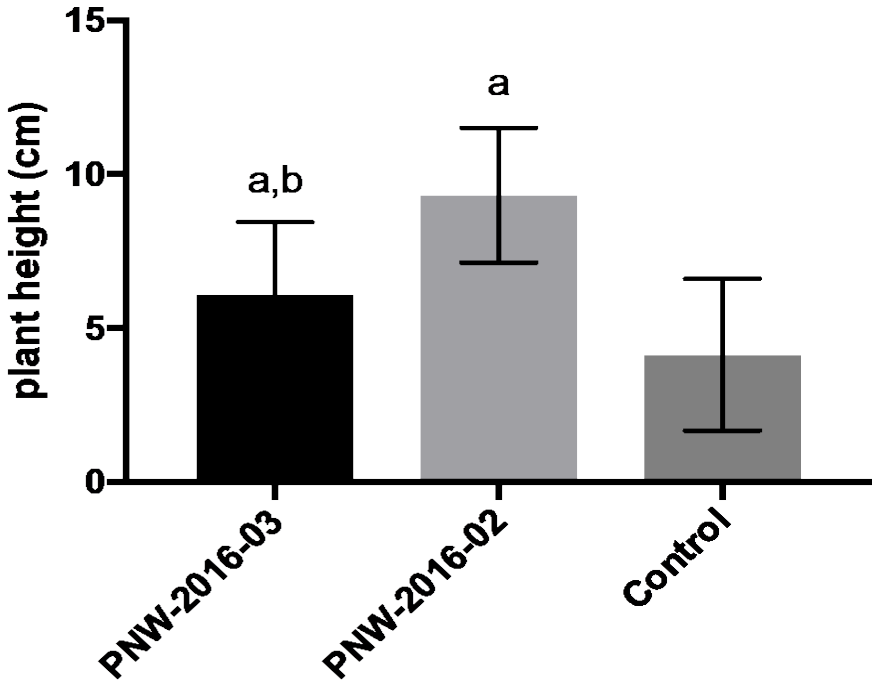

Figure 3: Bar graph of pooled plant height measurement data (n = 54) comparing PNW2016-02- and PNW2016-03-treated to that of the non-treated control plants. Letters indicate statistical significance among the three sets of data: a, $p < 0.0001$ to control; b, $p < 0.01$ to PNW2016-02.

3.3 Extracted DNA Concentrations from Experimental Treatments

Extracted genomic DNA concentrations from individual whole plants, roots and seedling leaves, from each treatment are given in Tables 2 and 3 below, while concentration values for the individual control plants are given in Table 4. Overall, we were able to successfully extract DNA for all plants from both the experimental and control treatments that were grown in the inoculation experiment.

Table 2: Extracted genomic DNA concentrations from individual plants treated with fungal endophyte PNW2016-02. Sample identification is indicated using an alphanumeric code for each plant corresponding to its initial position in the initial 96-well plate germination. The measured nucleic acid content of each sample is reported as ng/µL. The absorbance 260/280 ratio indicates the purity of each sample.

SAMPLE ID	Nucleic Acid Conc. (ng/µL)	Abs. (260/280)
YB13	4.50	1.81
RB11	3.40	1.94
RB6	3.30	2.09
RC10	3.50	1.76
YC3	5.70	1.62
YC9	6.60	1.58
YC6	5.50	1.52
YB1	3.50	1.93
YB7	5.10	1.69
RA12	4.70	1.72
GA9	3.80	1.75
YB18	3.30	1.95
RB15	7.70	1.57
GB10	9.80	1.52
RB16	5.10	1.62
RC7	4.00	2.05

Table 3: Extracted genomic DNA concentrations from individual plants treated with fungal endophyte PNW2016-03. Sample identification is indicated using an alphanumeric code for each plant corresponding to its initial position in the initial 96-well plate germination. The measured nucleic acid content of each sample is reported as ng/µL. The absorbance 260/280 ratio indicates the purity of each sample.

SAMPLE ID	**Nucleic Acid Conc. (ng/µL)**	**Abs. (260/280)**
GA16	6.00	2.00
RA13	3.50	1.61
RA8	4.20	2.11
GA2	7.00	1.8
YA5	5.00	1.92
GA4	5.40	1.79
GB3	8.70	2.15
YC17	5.40	2.04
GC1	8.80	1.96
GC14	4.30	1.94
GC4	5.50	1.71
RB17	7.10	1.88
GC5	4.50	1.67
YC11	4.50	1.85
YC13	5.30	1.81
GA7	5.70	2.00

Table 4: Extracted genomic DNA concentrations from non-treated control plants. Sample identification is indicated using an alphanumeric code for each plant corresponding to its initial position in the initial 96-well plate germination. The measured nucleic acid content of each sample is reported as ng/µL. The absorbance 260/280 ratio indicates the purity of each sample.

SAMPLE ID	**Nucleic Acid Conc. (ng/µL)**	**Abs. (260/280)**
RC8	4.10	2.10
YB2	3.80	1.61
YC15	4.80	2.14
RA6	6.00	1.74
GB9	4.10	1.98
GC15	5.60	1.89
YB2	5.70	2.16
GA14	4.40	2.08
RC8	6.60	1.88
GB12	4.70	1.98
YB17	5.50	1.79
RA15	4.10	1.78

3.4 Molecular Endophyte Detection

3.4.1 Pleosporales-Specific PCR Primers

The internal transcribed spacer region one (ITS-1) of the nuclear rRNA gene was successfully amplified in each of our endophytic *Stemphylium* strains, showing the expected band size in the 150 bp range in gel electrophoresis (see the positive PCR control in Fig. 7 for PNW2016-02 and data not shown for PNW2016-03). The designed primers also amplified ITS-1 in *Alternaria* (see the positive fungal PCR control representing that species in Fig. 7), a common endophyte species from the Pleosporales that is closely related to *Stemphylium*. The presence of pleosporalean fungal endophytes from within plant tissue was also confirmed using our specific primers (see the positive PCR control for leaf tissue in Fig. 7). Further, we confirmed that our specific primers did not amply ITS-1 when PCR assays were carried out on DNA extracted from fungal isolates of species outside of the Pleosporales (see the negative fungal PCR control in Fig. 7 depicting *Chaetomium*, PNW2016-04, from the order Sordariales) or un-inoculated spinach plants (see the negative PCR control for leaf tissue in Fig. 7).

3.4.2 PCR Phase of Endophyte Detection in Experimental Treatments

Amplification of ITS-1 from DNA extracted from individual plants of the inoculation treatment was also successful, with gel electrophoresis showing the expected band size in the 150 bp range for both for both PNW2016-02 and PNW2016-03 (see Figs. 5 and 6). As expected, we did not see IT-1 amplicons in PCR with our Pleosporales-specific primers in DNA extracted from un-inoculated plants from the control treatment in the final phase of the germination experiment. With the presence and persistence of PNW2016-02 and PNW2016-03 confirmed

from individual plants of the experimental treatment, we also quantified amplified nucleic acid concentration from the PCR phase of molecular detection (Tables 3 and 4).

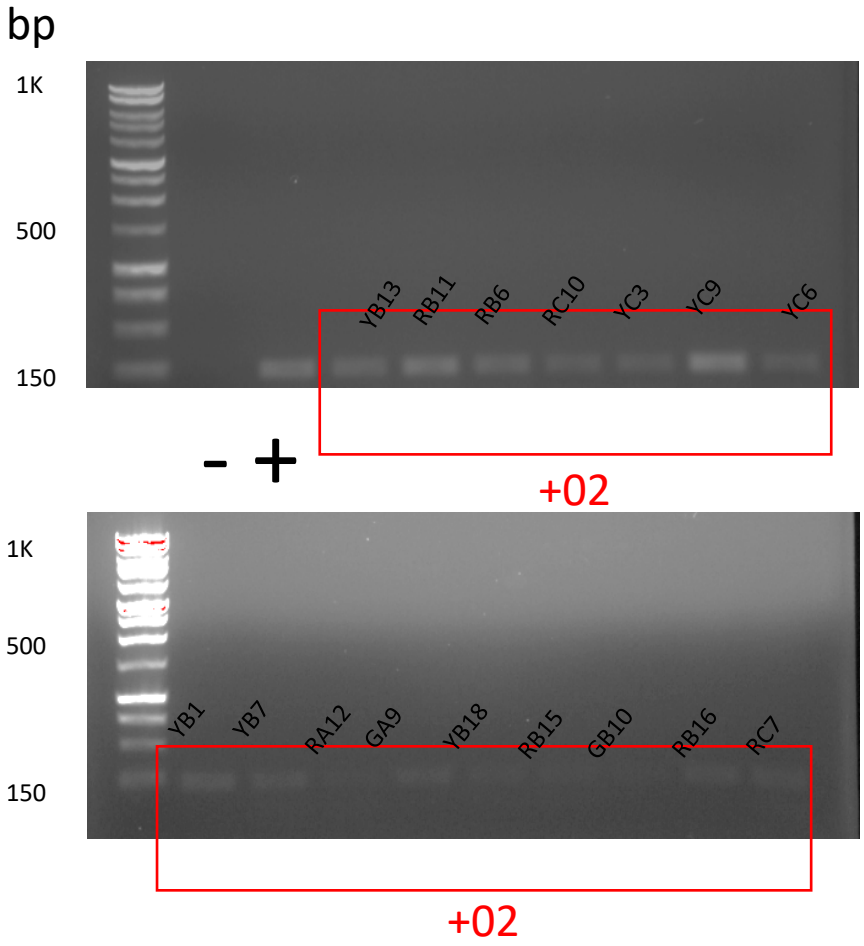

Figure 4: Gel images showing PCR ITS-1 amplicons from individual plants inoculated with PNW2016-02 in the final phase of the inoculation experiment (Top) Gel containing samples 1-7 in lanes #4-10. Lane 2 is a PCR negative control of un-inoculated plant tissue; Lane 3 is a PCR positive control of the fungal isolate PNW2016-02. (Bottom) Gel containing the remaining samples in lanes #2-10. Alphanumeric code of each sample is indicated above each amplicon band. Lane 1 in both gels contain a 1 kb DNA ladder.

Table 5: Concentrations of DNA from individual plants inoculated with the fungal endophyte PNW2016-02 in the final phase of the inoculation experiment following PCR amplification with our Pleosporales-specific primers amplifying the ITS-1 region of the nrRNA gene. Sample identification is indicated using an alphanumeric code for each plant corresponding to its initial position in the initial 96-well plate germination. The measured nucleic acid content of each sample is reported as ng/µL. The absorbance 260/280 ratio indicates the purity of each sample.

Sample ID	**Nucleic Acid Conc. (ng/µL)**	**Abs. (260/280)**
YB13	642.00	2.00
RB11	609.10	1.99
RB6	613.90	2.02
RC10	621.40	2.02
YC3	594.60	2.00
YC9	632.90	2.01
YC6	614.10	2.01
YB1	613.20	2.03
YB7	698.10	2.03
RA12	676.50	2.00
GA9	620.10	2.01
YB18	660.70	2.01
RB15	685.40	2.01
GB10	582.90	2.00
RB16	651.80	1.99
RC7	622.70	2.01

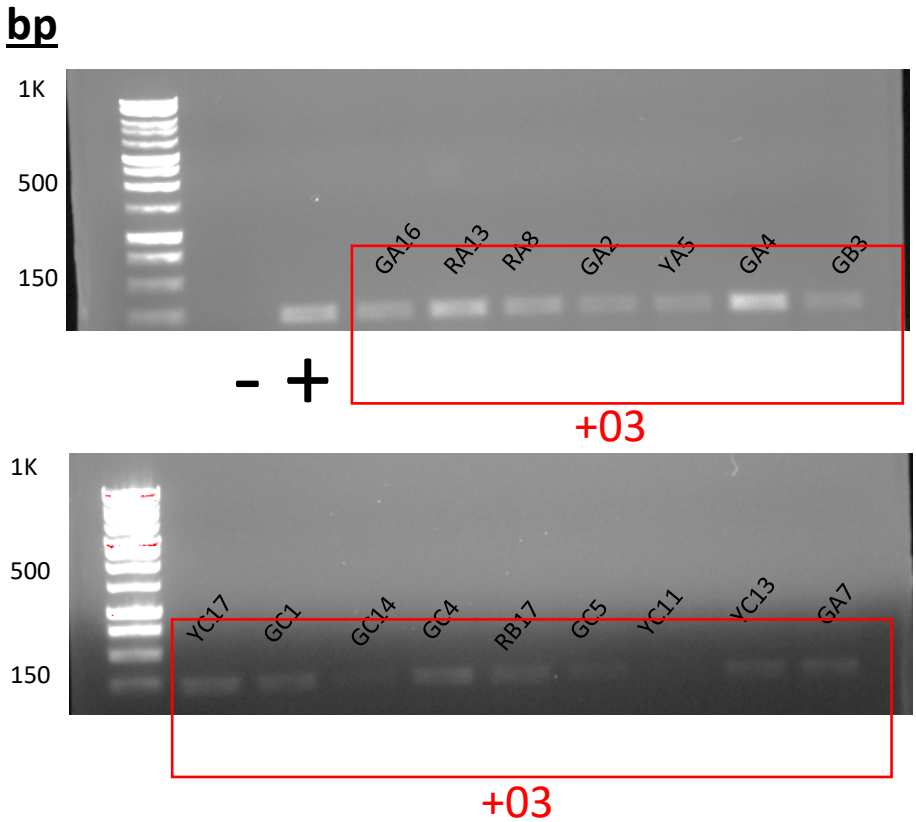

Figure 5: Gel images showing PCR ITS-1 amplicons from individual plants inoculated with PNW2016-03 in the final phase of the inoculation experiment (Top) Gel containing samples 1-7 in lanes #4-10. Lane 2 is a PCR negative control of un-inoculated plant tissue; Lane 3 is a PCR positive control of the fungal isolate PNW2016-02. (Bottom) Gel containing the remaining samples in lanes #2-10. Alphanumeric code of each sample is indicated above each amplicon band. Lane 1 in both gels contain a 1 kb DNA ladder.

Table 6: Concentrations of DNA from individual plants inoculated with the fungal endophyte PNW2016-03 in the final phase of the inoculation experiment following PCR amplification with our Pleosporales-specific primers amplifying the ITS-1 region of the nrRNA gene. Sample identification is indicated using an alphanumeric code for each plant corresponding to its initial position in the initial 96-well plate germination. The measured nucleic acid content of each sample is reported as ng/µL. The absorbance 260/280 ratio indicates the purity of each sample.

SAMPLE ID	**Nucleic Acid Conc. (ng/µL)**	**Abs. (260/280)**
GA16	641.90	2.01
RA13	608.90	2.02
RA8	628.10	2.04
GA2	652.90	1.98
YA5	719.10	1.99
GA4	663.70	2.03
GB3	640.60	2.02
YC17	673.90	1.99
GC1	605.60	2.00
GC14	709.60	2.00
GC4	663.10	2.01
RB17	689.90	2.01
GC5	705.50	2.01
YC11	662.80	2.04
YC13	711.80	1.96
GA7	643.80	2.00

3.4.3 *Stemphylium*-Specific Restriction Endonuclease Digest Assays

The restriction endonuclease *SnaBI* was used to distinguish *Stemphylium* endophytes from other pleosporalean fungi, such as *Alternaria*. Restriction endonuclease digest assays were performed on amplicons of fungal isolates *Stemphylium* and *Alternaria*, in addition to plant tissue from inoculated and un-inoculated plant tissue. Following the digest, gel electrophoresis showed *Stemphylium* isolates and tissue from plants inoculated with *Stemphylium* resulted in smaller *SnaBI* cut ITS-1 PCR amplicon fragments (~50 bp) as compared to *Alternaria* (~150 bp) as expected (Fig. 7). These results confirmed that the restriction endonuclease *SnaBI* is not only able to distinguish between *Stemphylium* and other pleosporalean fungal isolates, but that it can also be used to detect *Stemphylium* in inoculated plant tissue.

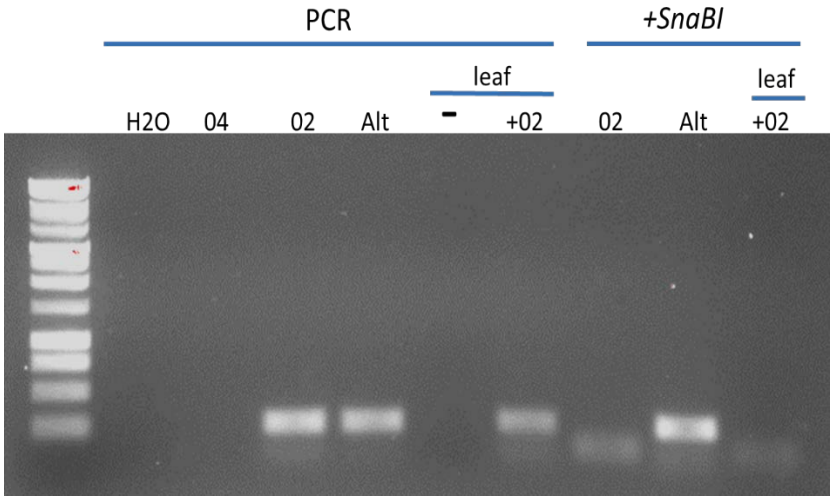

Figure 6: Gel image of PCR amplicons and restriction endonuclease digest of those amplicons. Lanes 4 and 5 show successful pleosporalean ITS-1 amplification from *Stemphylium* fungal isolate PNW2016-02 and fungal isolate *Alternaria*, respectfully. Lane 6 shows no amplification of ITS-1 from composite tissue of non-treated plants; whereas Lane 7 shows successful amplification of ITS-1 from composite tissue of PNW-2016-02-treated plants. Lane 8 shows the PNW2016-02 amplicon after digest with restriction endonuclease *SnaBI*. Lane 9 shows the *Alternaria* amplicon after *SnaBI* digest. Lane 10 shows 02-treated plant tissue composite amplicon after digest with *SnaBI*.

3.4.4 Restriction Endonuclease Assay Phase of *Stemphylium* Detection in Experimental Treatments

Detection of *Stemphylium* from individual plants of the inoculation treatment was also successful, with gel electrophoresis showing *SnaBI* cut ITS-1 PCR amplicon fragments of the expected size for both PNW2016-02 and PNW2016-03 (see Figs. 7 and 8).

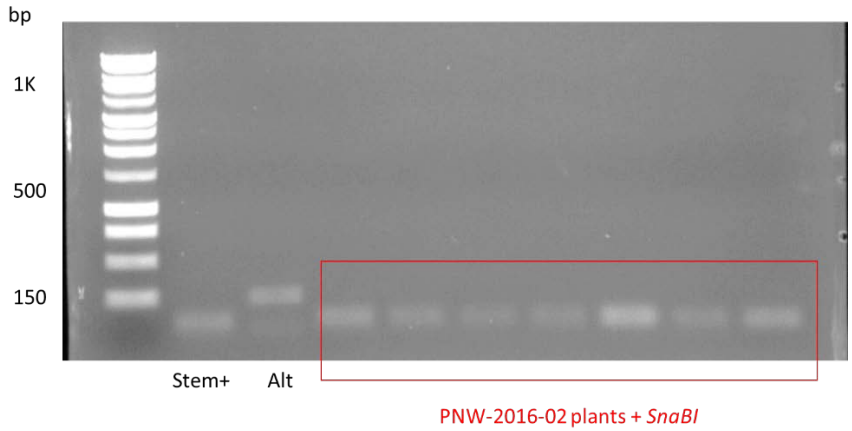

Figure 7: Gel image showing *SnaBI* cut ITS-1 PCR amplicons of individual plants inoculated with PNW2016-02 in the final phase of the germination experiment. Lane 2 is positive restriction endonuclease digest assay control of the fungal isolate PNW2016-02, while lane 3 is negative restriction endonuclease digest assay control of *Alternaria*.

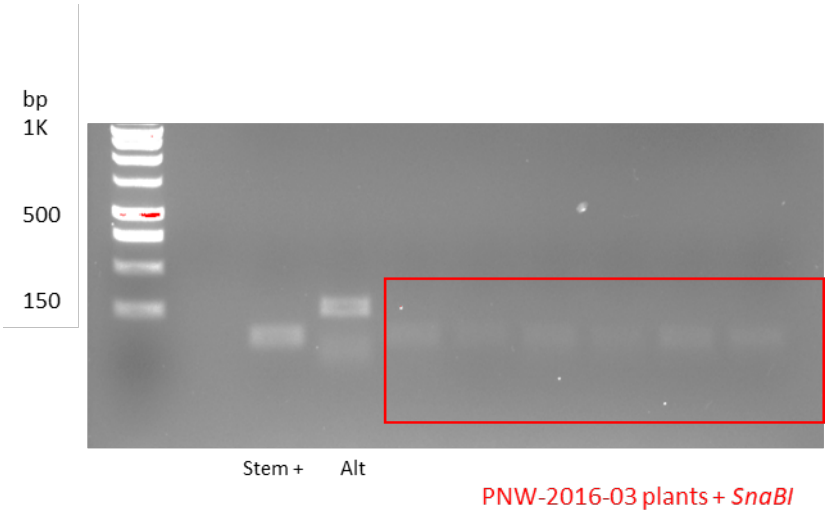

Figure 8: Gel image showing *SnaBI* cut ITS-1 PCR amplicons of individual plants inoculated with PNW2016-03 in the final phase of the germination experiment. Lane 3 is positive restriction endonuclease digest assay control of the fungal isolate PNW2016-02, while lane 4 is negative restriction endonuclease digest assay control of *Alternaria*.

3.5 Successful Inoculation in the Experiment

Over the course of our experiment, two of the 18 plants that were used in the inoculation experiment died in both experimental treatment groups. While factors that attributed to this plant death are unknown, we suspect that it may be related to stresses the plants were subjected to during the plant container transplanting process that was required for the final phase of the experiment. We did not notice any outward signs, such as leaf spot, on these plants suggesting that the *Stemphylium* endophytes were acting as pathogens. Both strains of our *Stemphylium* endophytes where successfully inoculated into spinach plants throughout the course of the experiment with a high rate of success, and overall we were able to achieve a 100% inoculation success using our methods for both the PNW2016-02 and PNW2016-03 strains.

CHAPTER 4: DISCUSSION

Our understanding of plant-endophyte relationships has been expanding due to innovations in molecular biology, allowing us to take a closer look into the plant endosphere. Being able to identify the microbial constituents of the plants we eat is important for many reasons, with food safety being at the top of the list. The overall goal of any research in this field is to examine plant-endophyte relationships at the molecular level, and be able to detect the internal colonization of the endophyte in question. The work here has shown progress toward this goal by developing a system for inoculating our *Stemphylium* endophytes into spinach seeds, grow the germinated seeds in an automated plant growth system, and design a molecular screening technique to detect successful inoculation and persistence of the fungal endophyte *in plantae*. The results of this work show that this aim was successful in all aspects.

4.1 Plant Height Measurements

Overall, we were able to develop a novel inoculation system to introduce *Stemphylium* to germinating seeds and detect the endophyte in the plant tissue. Upon completion of our experiment, we noted that the *Stemphylium* strain PNW2016-02 has the potential to not only improve germination rates of endophyte inoculated plants, but our results suggest it also has the capacity to promote plant growth in spinach plants. As seen in Figure 3, plants not treated with either endophyte (i.e., negative control) had diminished capacity for growth when compared to the endophyte inoculated plants.

The desirable attributes, the promotion of plant growth and seed germination success, seen in our study did not appear consistent for all *Stemphylium* strains as we did not see as dramatic of height increase for plants inoculated with strain PNW2016-03. This further suggests

we have isolated unique strains of *Stemphylium* or that closely-related strains can have considerably different effects on plant growth. Chemical analysis of secondary metabolites derived from each of these strains may point to some differences between PNW2016-02 and PNW2016-03. For instance, preliminary reports following HPLC analysis of the secondary metabolites from both strains were compared, revealing the presence of plant growth-promoting chemical compounds in PNW2016-02. The presence of these auxin-like compounds, such as 7-hydroxy-2',4',5'-trimethoxyisoflavone 7-O-glucoside and fulvine, appear to be highly expressed in the PNW2016-02 strain and align with the plant height increase seen in PNW2016-02-treated plants.

Chemical analysis of secondary metabolites derived from PNW2016-03 did not reveal the presence of these types of compounds, which is also consistent with the data from this study. Nevertheless, the enhanced plant height and increased seed germination success seen in PNW2016-02-treated plants was a serendipitous discovery, and represents attributes likely seen as desirable to commercial produce growers, thus they warrant further investigation. Being that *Stemphylium* species are widely known as a plant pathogen, there was no expectation of plant growth promotion.

Fungal species such as *Fusarium fujikuroi* and the endophyte *Porostereum spadiceum*, are both known to promote host plant growth via secretion of the metabolite gibberellin (Heden & Sponsel 2015; Hamayun et al. 2017), which ultimately increases the likelihood of their horizontal transmission to nearby plants. Perhaps the results seen here reflect a similar strategy for *Stemphylium* PNW2016-02. *Stemphylium* may provide plant growth stimulation initially to promote its own colonization, and alter metabolic function and health of the plant in mature leaves to aid in dispersal. The timing of the shift from being a beneficial endophyte to a pathogen

may be an important factor in allowing plants to mature before the onset of disease (D'Amico et al. 2006). Perhaps alternating from mutualist to pathogen is a mechanism to promote conidial spore dispersal once the host plant begins to show symptoms of disease caused by the endophyte, thereby facilitating spore transfer from fallen necrotized leaves. Future work will test this question by growing PNW2016-02-treated plants long-term to observe any pathogenic symptoms that may arise, and determine if this is a mechanism employed by this endophyte. *Stemphylium* colonization levels can be monitored to assess degrees of infection using our molecular detection technique.

4.2 Molecular Endophyte Detection

The work carried out in our experiment demanded that we develop a molecular method for detecting successful inoculation of our endophytic *Stemphylium* strains into the internal tissue of spinach plants. The Pleosporales-specific primers that we designed successfully amplified the ITS-1 region of the nrRNA gene in plants inoculated with both endophyte strains, and were able to successfully distinguish between *Stemphylium* and other fungal species in the Pleosporales using the *SnaBI* restriction endonuclease digest assay. Further, we were able to use our endophyte detection system to confirm the presence and persistence of *Stemphylium* in 100% of treated plants. This was true even in plant samples showing low extracted DNA concentrations prior to PCR. The measured nucleic acid concentration from individual plant tissue samples also likely included a high proportion of genomic DNA from the spinach plant. Assuming that most of the nucleic acid present in the sample was derived from the host plant, this would mean that the endophyte nucleic acid content in each sample is much lower when considering the plant-to-endophyte mass ratio. This suggests the primers developed here are highly effective and can be used as a part of a sensitive method for detecting *Stemphylium* in plants, and therefore could also

be used in additional studies, such as the detection of fungal species in commercially grown plants and/or environmental samples. Since *Stemphylium* is primarily known for its capacity to cause plant disease, such as leaf spot, our methods could be used as a tool to determine its early onset, which could be useful in commercial crop management.

4.3 Inoculation Success

Our results indicated that our plant-endophyte inoculation system was successful in reaching the proposed aim of this work, and to the best of our knowledge, our system also represents a novel approach for mass-inoculation of fungal endophytes into produce plants that has the potential to be scalable for commercial use. Although one study was found to use 96-well plate germination containers with agar media for germinating *Arabidopsis* for genotyping (Su et al. 2011), none were found using our method for inoculating plant seeds with a fungal endophyte.

4.4 Historical Relevance of Plant-Endophyte Symbiosis

We know that endophytes can range from being mutualistic to pathogenic in their relationship with a host plant, and seen here was a possible beneficial attribute by PNW2016-02, though this was unexpected due to *Stemphylium* being known as a fungal pathogen in other plants (Graf et al. 2015; Hanse et al. 2015; Olsen et al. 2018). By using plant-endophyte model systems, such as the one developed here, inquiries into the history of plant-microbe symbioses can be made to determine shifts in endophyte colonization levels, perhaps pointing to a specific time during the life of the plant when the endophyte may change symbiotic roles. Knowing the specific colonization strategies of plant endophytes, especially those that confer an advantage to the host plant, can be helpful in agriculture and crop management.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

With our efficient inoculation system, pleosporalean endophyte *Stemphylium*-like isolates PNW2016-02 and PNW2016-03 were successfully inoculated into pre-germinated spinach seeds, resulting in internalization and persistence in seedlings. PNW2016-02 and PNW2016-03 have shown *in vitro* inhibition of *E. coli* O157:H7, likely due to the production of fungal endophyte secondary metabolites with antimicrobial properties. A major goal of developing this plant-endophyte model is to examine the inhibition of human pathogenic *E. coli* O157:H7 by the *Stemphylium*-like endophytes PNW2016-02 and PNW2016-03 *in plantae*. Using the methods described here, the model can aid in determining the persistence of plant-introduced microbes, and can therefore be used to assess levels of *in plantae* colonization. With this ability, assays of internal endophyte interactions can be performed to determine the effectiveness of *E. coli* O157:H7 inhibition by fungal endophyte PNW2016-02 and PNW2016-03.

Another objective will be to observe long-range effects of PNW2016-02 colonization on host spinach plants. The results of the study described here showed height promotion in spinach plants inoculated with PNW2016-02. However, *Stemphylium* spp. are traditionally viewed as phyopathogens in numerous plant species, therefore plant growth promotion was not an expected outcome of these experiments. Further work will include determining at which point during growth inoculated plants begin to show symptoms of plant pathogenicity. Also examined will be whether the fungal endophyte is beneficial to the host only to aid in seedling growth to ensure its colonization, but then switch to a phytopathogenic role once it has established secure colonization.

Another future goal is obtaining genomic and metagenomic data to compare PNW2016-02 and PNW2016-03, in an effort to determine sequence differences. Preliminary HPLC analysis of secondary metabolites isolated from both PNW2016-02 and PNW2016-03 revealed the presence of chemical compounds associated with antimicrobial properties. In addition, the chemical analysis also showed the presence of plant growth promoting compounds in PNW2016-02, which aligns with results found in this study. Metagenomic analysis of our endophytes may reveal where these properties are on the genome.

Advancements in molecular and biochemical technologies have broadened our understanding of plant-endophyte relationships by allowing us to observe their interactions in nature and in the laboratory. It has become apparent that all plants are hosts to microbial communities that reside within the plant tissue. These endophytes may play crucial roles in plant health, and have likely co-evolved with plants to optimize their growth and survival. Some endophytes, such as *E. coli* O157:H7, exist within agricultural produce as human pathogens, causing illness in consumers. The novel plant-endophyte model developed here shows potential in the examination of *in plantae* inhibition of *E. coli* O157:H7 by the fungal endophyte PNW2016-02 and PNW2016-03.

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