Colonization of soybean (*Glycine max*) by the pathogen *Phialophora gregata* and endophytic fungi

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Abstract

Fungal pathogens, beneficial symbionts, and endophytes colonize plants and reduce, enhance, or have cryptic effects on plant productivity. My dissertation focused on colonization of soybean by the fungal pathogen *Phialophora gregata* and characterization of the endophytic fungal population within soybean. Soybean was used as a model plant because it is economically important, its colonization by *P. gregata* has well-defined latent and pathogenic phases, and endophytes within the stem were not characterized.

The first goal of this research was to investigate infection and plant responses during latent and pathogenic phases of disease development. Susceptible and resistant cultivars were infected with type A of *P. gregata* that causes stem and leaf necrosis or type B that causes only stem necrosis. Latent infection occurs after plants are colonized by a pathogen and remain asymptomatic. The number of vessels, leaf area, stomatal conductance, and yield of photosystem II (PSII) of infected and noninfected plants were determined. During latent infection, differences in the number of vessels was observed between susceptible and resistant plants, *P. gregata* was rarely observed in stems, and leaf area of susceptible plants was reduced by infection with type A. During pathogenic infection by type A, the resistant cultivar had fewer than 10% of vessels colonized and 20 to 25% more vessels than uninfected plants, while more than 70% of vessels were colonized in the susceptible cultivar and 50% fewer vessels were present compared to uninfected plants. During pathogenic infection by type B, more than 10% of vessels were colonized and no differences in vessel numbers were observed compared to the uninfected resistant plants. Type A did not reduce the leaf area of the resistant cultivars, but the leaf area of the susceptible cultivar was reduced by 80%. Type B reduced the leaf area of susceptible and resistant plants by 30%. Stomatal conductance was reduced 80% by types A and B in susceptible plants and by 40% in resistant plants. No differences in yield of PSII

were observed. Qualitative differences in colonization were observed during pathogenic infection using GFP and RFP-tagged isolates. Type B-RFP was observed in the primary xylem, while type A-GFP was observed outside of the primary xylem in the resistant cultivar. Whereas in the susceptible cultivar, PgA-GFP was in the primary xylem and PgB-RFP was limited to the interfascicular region. In summary, latent infection reduced the photosynthetic area of infected plants, but did not significantly modify their vascular structure and may lead to reduction of photosynthetic efficiency and increased susceptibility to biotic and abiotic stress. During pathogenic infection, resistant cultivars produced more vessels, restricted or excluded *P. gregata* from the vascular system, and reduced stomatal conductance and photosynthetic area. These responses may compensate for reduced vessel function and allow water movement.

The second goal was to determine the diversity of fungal endophytes in soybean stems. Stems from field-grown plants that were either treated or not treated with glyphosate were surface-disinfested, and fungal endophytes were assessed using culture-dependent (CD) and –independent (CI) methods. For the CD method, stem segments were dissected into an outer stem composed of the epidermal and vascular tissues and an inner stem composed of the pith tissues. Cultured fungi were grouped based on colony morphology and identified based on rDNA ITS sequences. For the CI method, DNA was extracted from stems and the ITS-region was amplified using fungal-specific primers, cloned, and sequenced for identification. More isolates were obtained from the outer than inner stems, and from the base of the stems compared to the apex. The most frequently isolated genera were *Cladosporium* (32%), *Phomopsis/Diaporthe* (15%), *Alternaria* (14%), *Fusarium* (11%), and *Phoma* (8%), The CD method detected more endophytic diversity (*H'* = 2.35) than the CI method (*H'* = 0.76). The most prevalent genus identified using the CI method was *Cladosporium* (83%). Soybean genotype influenced the diversity of endophytes more than glyphosate treatment. This research also suggests

soybean harbors an endophytic fungal population much less diverse than plants in the tropics and in polycultures.

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Literature Review: The mycobiota of soybean: colonization by the pathogen *Phialophora gregata* and endophytes

Soybean (*Glycine max* (L.) Merr.) is a versatile crop grown on over 70 million acres throughout the Central and Eastern U.S. In 2008, the estimated crop value was $27 billion in the U.S. Most soybeans produced are used for animal feed and oil, but other soy-based products are being developed to decrease our dependence on oil-based manufactured goods. Currently, 90% of newspaper ink is soy-based, and printing companies are beginning to develop soy ink for use in photocopiers and printers. The demand for soy-based plastics for the interior and exterior of automobiles and biodiesel is also increasing as the U.S. strives for autonomy from foreign oil. Soy-biodiesel is widely used to power the farm equipment of half of the U.S. soybean growers. As the demand for soy-based products increase, the incentives for a healthy, productive, and sustainable soybean crop also increase.

Given the monetary value and versatility of soybean, it is important to maintain its productivity. Each year the yield of soybeans is reduced due to the damage from pathogens. There are 40 known fungal pathogens of soybean throughout the world that can reduce yield by varying degrees depending on the environment, cultivar, and geographic location (21). Fourteen fungal pathogens are described as latent pathogens because symptoms are not observed until 6 or more weeks after infection (69). The latent period of soybean diseases is defined as “the interval from infection to display of macroscopic symptoms” (69). The latent period may be advantageous for pathogens by allowing them to increase their population within the host while not parasitizing or killing their host (69). Possible explanations for delayed symptom development include an environment not favorable for disease or an increase in host susceptibility as plants mature.

In Minnesota, the most prevalent latent pathogens known include: *Phialophora gregata* (Allington and Chamberlain) W. Gams (*Cadophora gregata* Harrington and McNew (29)), *Fusarium* spp., *Macrophomina phaseolina* (Tassi) Goidanich, *Phomopsis longicolla* T.W. Hobbs, *Colletotrichum* sp. and *Diaporthe phaseolorum* Cooke and Ellis. Irrigation, resistant cultivars, agronomic

techniques, and/or fungicides can partially manage many of these pathogens. However, these methods are often costly, potentially damaging to the environment, and are sometimes ineffective. Another approach to the management of latent pathogens that is being investigated is the use of endophytes. At its most basic definition the term endophyte means a fungus or bacterium within a plant, but the most common definition of endophytes are “microbes that infect host plants asymptomatically” and do not cause disease (63). This definition does not include latent pathogens since they cause symptoms of disease during a phase of their life-cycle. Recent investigations suggest endophytes are potential biological control agents (5,7,68), improve plant productivity (28,41), and enhance resistance to abiotic stress (48). Most research describes the interactions between endophytes and host plants as beneficial or mutualistic (5,7,28,36,37,48,51,68,71); however, the ecological function and role of most endophytes are unknown, and therefore it should not be implied that all endophytes are beneficial. There are reports of endophytes that negatively affect plant physiology and have the potential to become opportunistic pathogens when the environment is favorable (6,58,64).

Pathogens and endophytes routinely inhabit soybeans, but many unanswered questions remain about latent pathogens and soybean endophytes. The first goal of my research was to investigate the differences in colonization of soybean by fungi and physiology during latent and pathogenic infection. There are few published reports describing the physiological impact of latent infection on plants or the differences in colonization during latent and pathogenic infection (11,12). Latent and pathogenic infection was investigated using the pathogen *Phialophora gregata*, the casual agent of brown stem rot (BSR) of soybean. *P. gregata* is a is widespread throughout the North Central soybean growing region, is known to reduce yield, and typically has a latent period of 10 to 12 weeks. The second goal of my research was to determine the diversity of fungal endophytes in soybean. Endophytes in soybean stems in the U.S. have not been investigated and could potentially improve yield or could

enhance current management strategies. Furthermore, the possible negative impacts of endophytes in soybean production are also a concern.

Disease cycle and symptoms of brown stem rot (BSR)

P. gregata is a soil-borne pathogen with no known teleomorph stage (3). *P. gregata* infects roots 2 to 4 weeks after planting, but is typically not isolated from field plants until 8 to 9 weeks after planting, and symptoms do not develop before 10 weeks after planting (24,67). In the greenhouse, *P. gregata* has been recovered from the apex of plants one hour post inoculation (73). Because of the speed by which this occurred, conidia may readily travel throughout the vascular system and systemically colonize plant tissues during latent infection (72,73). Latent infection transitions to pathogenic and symptoms appear during mid reproductive growth stages of soybean when the environment is favorable and host is susceptible. During pathogenic infection, symptoms appear during R3 and progress as plants mature, often causing premature defoliation. Typical symptoms of BSR include interveinal chlorosis and necrosis of leaves, leaf abscission, and internal stem browning of vascular and pith tissues (3). Symptoms of BSR differ depending on whether plants are infected with type A or B of the pathogen. Infection of soybean with type A (formerly type I) of *P. gregata* typically causes foliar and internal stem symptoms (22,32). Vascular discoloration is sometimes observed when plants are infected by type B (formerly type II), but foliar symptom severity is typically less than 10% (32). Internal stem browning can also be attributed to other fungi that colonize soybean, including *Verticillium dahliae* (74), *Fusarium oxysporum* (19), and *Stilbella* sp. (57), but none of these fungi cause foliar symptoms similar to BSR.

BSR symptoms are attributed to phytotoxic metabolites produced by *P. gregata*. Five metabolites known as gregatin A, B, C, D, and E are associated with symptoms of BSR and reduced electron transport in the cytochrome *b/f* complex during photosystem II in soybean (25,26,39,65). Interveinal leaf necrosis and internal vascular browning of the stem in mung bean and adzuki

bean are associated with gregatin A, C, and D (39). Only leaf necrosis (no vascular browning) of mung bean and adzuki beans was associated with gregatin B and E (39). Seedlings of soybean and kidney bean cut at the base of the plant and immersed in solutions of each gregatin showed foliar symptoms of necrosis and chlorosis similar to adzuki and mung bean, but no vascular browning was observed (39). Soybean trifoliates placed in a stem extract collected from plants inoculated with type A expressed interveinal necrosis, chlorosis, and wilting 3 days after treatments (25). Similar experiments using a type B isolate did not result in interveinal necrosis or wilting (25).

Effects of BSR on soybean yield

Yield loss due to BSR varies depending on the type of the pathogen and severity of disease, but typical estimates range from 10 to 30% (53). The extent of the yield loss associated with type A and B is under investigation. Yield loss has been reported to be greater when plants have both foliar and stem symptoms compared to plants with only vascular browning, but none of this research has specifically looked at type A and B (23,24,53). Recent investigations in which plants were inoculated with either type A and B indicate both cause a yield loss (33,47).

Understanding the physiological and anatomical basis cause for yield loss due to *P. gregata* infection is challenging. BSR may reduce photosynthesis as a result of foliar symptoms, or it may disrupt water flow through the vascular system due to internal stem damage. Chamberlain (1957) used a plant extract derived from soybean infected with *P. gregata* to study water flow in soybean. Water flow was reduced in plants treated with extract from diseased plants compared to extract from healthy plants (14). The functioning of the vascular system was further assessed by measuring stomatal conductance, transpiration, and leaf water-potential to determine the physiological responses of susceptible and resistant soybean during pathogenic infection when inoculated with type A (30). Beginning at R2, stem conductance declined and

continued through R5.5. However, transpiration and stomatal conductance increased, resulting in increased water loss (30). In similar experiments, transpiration decreased when plants were infected with *P. gregata*, but stomatal conductance did not differ between infected and uninfected plants in the field (77). Whether these adverse affects are the result of vascular occlusion or a phytotoxin remains unknown. The decrease in water flow to the soybean sinks can be a significant factor in the development of soybean seed. Soybean subjected to water stress can maintain seed number but have a decrease in seed size (18,38,56,70).

Diagnosis and management of BSR

Symptoms can often be used to diagnosis BSR in the field, but are not always obvious. Foliar symptoms are usually not observed with type B, and sometimes not with type A. Foliar symptoms can also easily be confused with symptoms of sudden death syndrome, so proper diagnosis is dependent on looking for internal BSR stem symptoms. In the lab, isolation or molecular detection can be used to detect the pathogen. *P. gregata* can be isolated from stem tissue using PDA or semi-selective PGM, but *P. gregata* is slow-growing and easily outgrown by faster growing fungi. Standard PCR (sPCR) and real-time quantitative PCR (qPCR) are more rapid, efficient techniques for lab diagnosis, detection and quantification of the pathogen in plant tissue (15,46). Types A and B can be distinguished by sPCR based on variation in the intergenic spacer region (IGS) of the nuclear rDNA (15).

Management of BSR has been extensively studied since the first report of the disease in Illinois in 1948 (3). Management is a coordinated effort that includes host resistance, crop rotation, and tillage. No-till or conservation tillage has become more popular to growers because it reduces loss of soil moisture, reduces soil erosion, decreases labor, and reduces fuel costs for machinery; however, *P. gregata* survives as a saprophyte on the soybean residue that remains in fields after harvest (43). The survival of *P. gregata* on soybean

residue depends on tillage and resistance or susceptibility of the plant tissue (2,35). Severity of BSR in no-till systems is reported to be 38% greater compared to conventional till (1). The accumulation of soybean residue in no-till fields increases the persistence of *P. gregata* (2,35). In addition to conventional tillage, resistant cultivars are used to manage BSR . Three genes designated as *Rbs1*, *Rbs2*, and *Rbs3* are reported to confer major gene resistance to BSR (27,78). A major QTL has also recently been identified in PI 88788 (61). Resistant cultivars have lower foliar and stem symptom severity and also decrease inoculum potential (35,54). A third management technique to decrease BSR is crop rotation. Crop rotations of resistant cultivars and corn decrease inoculum potential (1).

An emerging approach to manage *P. gregata* is biological control. A bacterium that suppressed growth of *Fusarium oxysporum* and *P. gregata* and also stimulated root growth was isolated from the rhizosphere of soybeans with root rot (76,80). Biological control alone may not be sufficient to control BSR, but has potential if it is used in combination with another control strategy. More potential biological control agents could reside in the rhizosphere or within soybean roots, stems, or leaves, but are unknown. Therefore, the rhizosphere and plant endophytic microbial community merits further investigation.

Beneficial and negative outcomes of endophytic and pathogen-like infection

Endophytes have the potential to act as biological control agents of pathogens or to improve plant growth and productivity, or some endophytes could be potential latent or opportunistic pathogens. Studies in South America and Asia provide some evidence for the potential positive affects of endophytic bacteria in soybean production (34,41). High frequencies of bacteria able to produce indoleacetic acid were isolated in both studies, and have potential to improve plant growth. The most common bacterial endophytes included *Pseudomonas, Ralstonia*, and *Enterobacter* (41). In addition to these potential

beneficial bacteria, some of the most frequently isolated fungi from soybean may also have positive functions in soybean. An unpublished report provides some evidence that a common soybean endophyte, an *Acremonium*-like fungus (probably *Plectosporium tabacinum* (teleomorph: *Plectosphaerella cucumerian* (Lindf.) Kleb (16)), may reduce severity of white mold, however, this fungus also potentially causes symptoms similar to the disease brown stem rot ((52) C. Grau and J. Kurle, 1999 unpublished data). *Acremonium* sp. causes vascular and pith discoloration, but no foliar symptoms (52).

Endophyte infection can also change plant physiology in some plant systems. For example, *Theobroma cacao* (cocoa) was inoculated with multiple species of endophytic fungi and conductance of the leaves was evaluated. The conductance of leaves of *T. cacao* was reduced by 90 to 99% during periods of drought, however, inoculation with endophytic fungi increased leaf conductance, which may enhance drought stress resulting in low water potential, embolisms, and/or leaf abscission (6). In another investigation, photosynthesis was evaluated in banana (*Musa acuminate*) and corn (*Zea mays*) infected with *Colletotrichum musa* and *Fusarium moniliforme*, respectively (64). These fungi are pathogens, but are also known to infect and colonize plants asymptomatically. No symptoms of infection were observed on corn or banana, but photosynthesis was reduced compared to uninfected plants (64). The banana-*C. musa* and corn-*F. moniliforme* interactions are described as endophytic throughout this paper, but latent colonization may be a better description since photosynthesis is reduced.

Endophytic and pathogenic differences in colonization

A delicate balance between the host and fungus must occur for asymptomatic colonization, and a niche within the plant must be available for the microbe to colonize (49). Fungi in corn were found to colonize a different niche than bacteria (20). Fungi were more frequently isolated from the stem than from leaves, and from the mid to upper level of the plant (20). In contrast,

bacteria were more frequently isolated from stalk tissues near the soil line. In addition to differences between apical and basal colonization for fungi and bacteria, endophytic and pathogenic microbes colonize different areas within the plant (9,49). Bacterial endophytes typically colonize the apoplast and do not cause plant stress or impair function. One could think that the xylem of plants are opportune habitats for endophytes because the transfer of water and solutes within the xylem provide endophytes with a continuous food supply (49). However, xylem vessels are not ideal habitats for bacterial endophytes. Vessels invaded by bacteria are typically non-functioning, partially due to development of gas embolisms (49). The xylem also has a defense response, in which xylem parenchyma cells secrete mucilaginous and phenolic materials into the vessels, which restrict microbe movement and cause a decrease in water conduction. The intercellular spaces of plants is a more ideal environment for endophytes. The volume of these spaces is much greater than that of the vascular tissues, and typically have a higher solute (sugar) concentration than the xylem vessels (49).

Our understanding of how the plant tissues are infected and colonized by pathogens and endophytes has improved with the use of transmitted and fluorescent light microscopy (31,44,59,60,62,75,79). For example, *Fusarium verticilloides* (formerly *F. moniliforme*) is both an endophyte and pathogen of corn (8,79). During endophytic colonization hyphae are limited to intercellular colonization, and lignin deposition in the stem of corn seedlings increases, possibly creating a barrier to pathogenic infection (8,79). Pathogenic infection resulted in inter and intracellular hyphae, as well as reduced plant growth (8,79). These investigations were conducted using transmitted light microscopy, which is unable to distinguish different pathogens and endophytes in plants. Fungi that have been transformed to express fluorescent proteins are useful in enhancing our understanding of inter- and intracellular colonization. Green and red fluorescent proteins (GFP/RFP) have been modified for stable expression in fungi, and are strongly expressed with the proper promoter (4,13,44). GFP is

the most widely used reporter that can be used to gather detailed information on infection and colonization in plants. For example, the fungus *Colletotrichum acutatum* J.H. Simmonds, was transformed to express GFP to study its pathogenic association with strawberry, and its non-pathogenic survival on other hosts (31). Appressoria of *C. acutatum* failed to germinate and infect non-host plants, whereas appressoria quickly developed and penetrated cells intracellularly and caused necrosis of strawberry tissues. As an endophyte *C. acutatum* can invade tissues of hosts, but is restricted to intercellular spaces and plants remain asymptomatic (31).

Herbicide effects on soybean endophytes and pathogens

Colonization of soybeans by some endophytes and pathogens is modified by the use of herbicides. Greater than 90% of the soybeans grown in the U.S. in 2008 were resistant to the herbicide glyphosate. Glyphosate inhibits production of aromatic amino acids, and therefore, inhibits production of lignin precursors, some phytoallexins, and other phenolics important in plant disease resistance. The application of glyphosate may also weaken plants making them more susceptible to opportunistic or latent fungi. Glyphosate is degraded within the plant or is translocated to the roots and released into the soil. The effect of glyphosate on the microbial community within plants and in soil has been widely studied and is variable. For instance, glyphosate increased colonization of soybean roots by *Fusarium* sp. and *Pythium,* and the overall population of *Fusarium* and *Pythium* in crop residue was increased, which can potentially increase damping-off of seedlings (17,40,55). Disease severity of sudden death syndrome and isolation of *F. virguiliforme* also increased after application of glyphosate (66). In contrast, *in vitro* fungal growth of *F. virguiliforme* was reduced with glyphosate amended media (66).

The effects of glyphosate on beneficial organisms such as mycorrhizae, rhizobia, and bacterial endophytes have also been studied. Growth of arbuscular mycorrhizae and *Bradyrhizobium* were inhibited in culture medium

containing glyphosate, but glyphosate had no effect on *Bradyrhizobium* nodulation or mycorrhizal colonization in greenhouse studies (45). Endophytic bacteria were analyzed from soybean leaves, stems and roots that had received a pre-planting application of glyphosate to the soil (42). Denaturing Gradient Gel Electrophoresis (DGGE) revealed that some species were exclusive to plants with pre-planting glyphosate, while other bacteria were exclusive to plants without glyphosate. In addition, only two species of bacteria were recovered from soybean tissue plated onto a glyphosate-enriched medium indicating they may be less sensitive to glyphosate than other species.

Physiological methods used to study plant-microbe interactions

Studying the impact of invasion by pathogens, endophytes, and mutualists on plant physiology can improve our understanding of symptom development and progression of disease. Chlorophyll fluorescence is a common measure of plant physiological stress because data are easy to collect, changes in photosynthesis can be detected before symptoms are visible, and sampling is not destructive. The principle behind measuring photosystem II (PSII) using chlorophyll fluorescence is based on understanding that the electron and light harvesting capacity of PSII is not infinite, and there are three fates of light energy. Light energy can be dissipated as heat (~70%), it can drive electron transfer from the PSII reaction center to the primary electron acceptor, quinone A (~30%), or it can be re-emitted as light in the form of chlorophyll fluorescence (~2%). The three fates compete, and theoretically add up to “1” so that changes in photosynthetic yield are related to changes in photochemistry, heat dissipation, and fluorescence (1 = P + H + F).

Changes in fluorescence and photosynthesis occur under different environmental conditions. For example, when leaves are transferred from darkness into the light, the reaction centers and the quinone electron acceptors of PSII are quickly filled, and therefore “closed”, causing a decrease in photochemistry and an increase in fluorescence and heat dissipation. After a

few minutes, the fluorescence decreases, or is “quenched”, due to two different factors. First, enzymes involved in the carbon fixation reactions (Calvin Cycle) are activated in the light and electrons are transported away from PSII to PSI; and also energy is dissipated as heat at a faster, more efficient rate.

Chlorophyll fluorescence is typically measured using a pulse amplitude modulator (PAM). Fluorescence is highest when photochemistry and heat dissipation are lowest, and therefore, changes in fluorescence reflect changes in photochemistry and heat dissipation. For my dissertation experiments, I gathered data on chlorophyll fluorescence and calculated quantum yield of PSII from dark adapted plants. In dark-adapted leaves, a high-intensity, short-duration light (3 µs) is used to close all PSII reaction centers, and the contribution of photochemistry is assumed to be zero as all reaction centers are closed, (P = 1 - F - H). The maximum fluorescence (Fm) is determined in conjunction with the minimum fluorescence (Fo) in the absence of actinic light, and is used to determine Fv. Then the photosynthetic efficiency or yield (Fv/FM) in dark adapted leaves is determined (Fv/Fm = (Fm – Fo)/Fm).

Another non-invasive approach to investigate physiological stress is to measure stomatal conductance. Stomata are openings on the abaxial and adaxial surface of leaves that allow for exchange of CO_2, O_2, and water vapor between the plant and the environment. Stomata are open or closed based on humidity, light, internal CO_2 concentration, water stress, and pathogens. Stomatal conductance (or resistance) is measured by using a porometer. The porometer is calibrated by using a perforated plate with holes of known size and known conductance. After calibration, a leaf is inserted into the chamber and the chamber is flushed with dry air. After a pre-selected state of low relative humidity is achieved, the porometer measures the time it takes for a leaf to release water vapor to change the relative humidity in the chamber. The values obtained from the calibration and the leaf are used to derive stomatal conductance of the leaf. In potato, differences in gas exchange were observed before symptoms of *Verticillium* wilt (potato early dying) were apparent, but

differences in inoculated and non-inoculated plants were not significant. During pathogenic infection, potato leaves were often stunted and gas exchanged was reduced (11).

Stomatal conductance and chlorophyll fluorescence were recently used to study water stressed and non-water stressed *Parthenocissus quinquifolia* (a deciduous vine) infected by the xylem-limited bacterial pathogen, *Xylella fastidiosa*. In high water (HW) or low water (LW) plants, no differences in stomatal conductance were observed in the apex of infected and uninfected plants. In leaves near the base of plants, no differences in conductance were observed during early infection, but conductance was reduced by as much as 50% as symptoms began to worsen in LW plants (50). Similarly, no differences in photosynthesis efficiency were observed in HW or LW plants in the apex of infected and uninfected plants. However, photosynthetic efficiency declined as symptoms were observed in the lower leaves of LW plants. In general, plants under water stress due to stomatal closure also have reduced photosynthesis (10).

Research goals and significance

The incentives for growing a healthy and productive soybean crop are both monetary and ecological. Plant-fungal interactions are ideal systems to study basic biological and ecological principles. My main interest is in how beneficial, pathogenic, and endophytic fungi colonize plants, and the effect of fungal colonization on the physiology, anatomy, and productivity of plants. The objectives of my research were to determine if the physiology and colonization of soybeans is modified during latent and pathogenic infection by *P. gregata*; and to determine the identity and diversity of the endophytic community within soybean. The interaction between soybean and *P. gregata* is an excellent model to provide detailed analysis of latent and pathogenic lifestyles. Endophytes have potential to make our current agriculture system more sustainable, but need to be thoroughly investigated to identify potential benefits

and because they also potentially have negative effects. Utilization of endophytic fungi to prevent pathogen damage and increase plant productivity is dependent on our understanding of these interactions and our ability to potentially manage the endophytic populations in the plant. Ultimately, some of these interactions may be useful for: improving biomass production and growth of plants used for biofuels, enhancing plant productivity in agronomic systems without the addition of chemical amendments, and improving phytoremediation.

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Chapter 1: Result of latent and pathogenic infection by *Phialophora gregata* on soybean chlorophyll fluorescence, stomatal conductance, and leaf area

Many plant diseases have a poorly understood latent phase in which macroscopic symptoms of disease are delayed. The interaction between soybean and the yield-limiting fungal pathogen, *Phialophora gregata* is an excellent interaction to study because it has an extensive latent phase that begins 2 to 3 weeks after planting and continues until symptoms of disease appear during reproductive growth stages. The objective of this study was to determine if latent or pathogenic infection adversely affects leaf area, stomatal conductance, or photosynthesis of resistant and susceptible cultivars. Plants were inoculated with either type A of *P. gregata*, known to cause leaf and stem symptoms, or type B, which only causes stem symptoms. At 3 weeks post inoculation (WPI), no differences in leaf area of resistant plants was observed with or without inoculation. The leaf area of susceptible plants infected with type A was reduced by 30%, whereas leaf area was not reduced during latent infection by type B. Neither stomatal conductance nor yield of photosystem II (PSII) was adversely affected by type A or B during latent infection of resistant or susceptible plants. During pathogenic infection, type A did not reduce the leaf area of the resistant cultivars, but the leaf area of the susceptible cultivar was reduced by 80%. Type B reduced the leaf area of susceptible and resistant plants by 30% during pathogenic infection. The stomatal conductance of susceptible plants infected by type A and B was reduced approximately 80% and of resistant plants by 40%. The yield of PSII was not affected during pathogenic infection. These results suggest latent infection reduces photosynthetic area, while pathogenic infection reduces photosynthetic area and stomatal conductance, but yield of PSII is not reduced during latent or pathogenic infection. *P. gregata* colonizes intercellularly during latent infection and moves into the vascular system during pathogenic infection, and may disrupt flow through the vascular system without modifying the photosynthetic machinery.

INTRODUCTION

Many plant diseases have a poorly understood latent phase between infection and the development of visible symptoms. The latent phase may result from an unfavorable environment or a host that is not susceptible until its resources are shifted from defense and vegetative growth to reproduction. Delayed symptom development could be an advantage for the pathogen and a disadvantage for the host. During latent infection, pathogens may reproduce and colonize the plant, which may hypothetically weaken the plant, predispose it to further infection, or modify plant physiological processes and decrease growth and productivity (19). The effects of latent infection on plant physiology have not been studied.

There are 40 known fungal pathogens of soybean that can reduce yield by varying degrees depending on the environment, cultivar, and geographic location (6). Fourteen fungal pathogens are described as latent pathogens, and those that are common in the Midwest include: *Phialophora gregata* (Allington and Chamberlain) W. Gams (*Cadophora gregata* Harrington and McNew (9)), *Fusarium* spp., *Macrophomina phaseolina* (Tassi) Goidanich, *Phomopsis longicolla* T.W. Hobbs, *Colletotrichum* spp. and *Diaporthe phaseolorum* Cooke and Ellis (19). In this study, the interaction between soybean (*Glycine max* L. Merr) and *Phialophora gregata*, a common soybean-infecting fungus that causes brown stem rot was used to investigate the physiology of plants during latent and pathogenic infection. The interaction between soybean and the brown stem rot (BSR) pathogen, *P. gregata*, is an excellent model system because *P. gregata* can reduce soybean yield at least 30% and has a latent phase that can last 8 to 10 weeks, which allows ample time for investigations on soybean physiology (16). The interaction is also ideal to study because the transition from latent to pathogenic is consistent in the field, and the first symptoms of BSR typically appear at the R4 growth stage or 10-12 weeks after planting.

Symptoms of BSR observed during the pathogenic phase of the infection can either be associated with the leaves or stem, and differ depending on whether plants are infected with type A or B of the pathogen. Foliar symptoms are interveinal chlorosis and necrosis followed by leaf abscission. Stem symptoms consist of browning of vascular and pith tissues (1). Infection of soybean with type A causes foliar and stem symptoms (11). Plants infected with type B always develop stem symptoms, but typically have less than 10% foliar symptom severity (11). If disease is severe with either type, 100% of the stem can be necrotic, which suggests water relations of the plant may be modified. During pathogenic infection, stomatal conductance of plants infected with *P. gregata* increased in the greenhouse and resulted in increased water loss, but there were no differences in stomatal conductance between infected and uninfected plants in the field (10,20). Leaf water potential was also reduced in greenhouse and field studies, but yield was not significantly reduced in the field (10,20). These studies were conducted during the pathogenic phase when symptoms were visible. The influence of *P. gregata* infection on soybean physiology and growth during the latent phase has not been studied.

The main goal of this study was to determine if soybean physiology and growth is modified due to latent and pathogenic infection by *P. gregata*. We investigated growth, stomatal conductance, and photosynthetic efficiency of resistant and susceptible soybean cultivars infected with types A and B of *P. gregata* in the field and in controlled environments. The etiology of latent infection is not well understood, and may be important for understanding the ecology of infected plants as well as reveal information useful for development of effective control measures during the latent phase of disease.

MATERIALS AND METHODS

Stomatal conductance and leaf area. The susceptible soybean cultivar, AG2107, and the resistant cultivar, AG2106, were grown in 1 gallon pots containing LB2 professional potting mix (Sun Gro Horticulture, Bellevue,

WA). When plants reached the VC-V1 growth stage, they were thinned to one plant per pot and ca. 100 µl of *P. gregata* inoculum was injected into the base of the stem using a 18G1 hypodermic needle (BD, Franklin Lakes, NJ) and a 10 cc syringe. Inoculum was produced by growing a type A isolate from Olmsted County, MN (PgMNRa) and a type B isolate from Ramsey County, MN (PgMNCb) in soybean seed broth (12 g of susceptible soybean/100 ml water) for 21 days. The fungal growth was macerated with a PowerGen Model 1800 homogenizer (Fisher Scientific, Pittsburgh, PA), conidia and mycelia fragments were counted, and inoculum was adjusted to 5.0 x 10^6 CFUs ml^{-1}. The inoculation treatments were types A, B, and a control consisting of sterile soybean seed broth. Three separate experiments were set-up at different times in a randomized complete block design with five replications of each treatment in a greenhouse maintained with a 14 hr photoperiod and day and night temperatures of 22℃ and 18℃, respectively, from Octobe r 2006 to May 2007. Marathon and Gnatrol *(*Valent U.S.A Corp, Walnut Creek, CA) were applied as needed to control thrips and fungus gnats.

Stomatal conductance and leaf area were measured weekly beginning 1 week post inoculation (WPI) and continued to 9 or 10 WPI. Stomatal conductance readings were taken from 0900 hours to 1200 hours, approximately 1 to 2 hrs after plants were watered to saturation. An AP4 porometer (Delta-T Devices, Cambridge, United Kingdom) was calibrated and the conductance of the adaxial and abaxial surfaces of the youngest, newly expanded center leaf of the trifoliate was measured. The approximate leaf area was determined by measuring the length and width of the center leaf of each fully expanded trifoliate on each plant.

Chlorophyll fluorescence. Field experiments were planted on 11 May 2007 and 19 May 2008 in field locations K2 (2007) and C1 (2008) with no history of soybean production for the preceding 5 years on the St. Paul Minnesota Agriculture Experiment Station. Two susceptible cultivars, AG2107 and Garst 1824, and two resistant cultivars, IA 2008 and AG2106 were planted

at a rate of 150,000 seeds per acre in four-row plots that were 4.5 m long and spaced 0.8 m apart. The fields were set-up in a RCB design with four replications. One center row per plot was inoculated with 100 µl of 2.0 x 10^6 CFU ml^{-1} of the type A or B treatment or control at the V1 to V2 growth stage in June 2007 and 2008 as described above. The two outer rows of each plot were border rows.

Growth chamber experiments were conducted from October 2008 through April 2009 using the same four cultivars. Three plants were grown in 15 cm pots containing LP5 mix (Sun Gro Horticulture, Bellevue, WA) and 5 cc^3 of osmocote classic (Scotts Miracle Gro, Maryville, OH). Plants were inoculated at the VC-V1 growth stage with 5.0 x 10^6 CFU ml^{-1} of types A, B, or control as described above, and five replications of each treatment were arranged in a RCB design. Plants were grown with a 12 hr photoperiod and day and night temperatures of 22℃ and 18℃, respectively.

A pulse amplitude modulated fluorometer (MINI-PAM, Heinz Walz, Effeltrich, Germany) was used to determine the photochemical efficiency also known as the quantum yield of PSII (Fv/Fm) for dark-adapted leaves. Readings were taken in the field between 0400 and sunrise from the youngest, fully-expanded leaflet of ten randomly selected plants in each plot every 10 days beginning in the latent phase on 15 July and continuing through the pathogenic phase in mid August in 2007 and 2008. In the growth chamber, plants were dark-adapted for 8 to 10 hrs before assessment and weekly data collection began two WPI and continued until 8 WPI. At 2 and 3 WPI, data were collected on the youngest, fully expanded leaf of each plant. At 4 to 5 WPI, data were collected on the youngest, fully-expanded leaf and two mature leaves. The oldest, youngest, and three randomly tagged leaves were measured from 6 to 8 WPI.

Symptom and pathogen assessment. In the greenhouse and growth chamber, BSR foliar severity was rated weekly from 5 WPI to 9 or 10 WPI, and foliar severity in the field was determined for each plot at the R7 growth stage.

Foliar severity was determined by counting the number of diseased trifoliates and dividing that number by the number of healthy trifoliates and multiplying by 100. Stem symptom severity was rated typically at R5/R6 growth stage in the growth chamber and greenhouse, and at the R7 stage in the field. Stems were split longitudinally and the percent of the stem length with browning was measured to determine severity. Five randomly selected stems were rated per plot in the field, and all plants were rated in the controlled environments.

The presence or absence of *P. gregata* was determined in all experiments by conducting PCR assays with the lower 10 to 15 cm of stem. The stems from each pot in controlled environments were pooled into a single sample and five plants from each plot in the field were collected and pooled. Stems were dried for 48 hrs at 33℃, ground with a Wi ley mill, stored at -20℃, and DNA was extracted and *P. gregata* detected with a specific, standard PCR assay (3,14).

Data analysis. The mean foliar and stem symptom severity for all plants evaluated per replication was calculated and used for analysis. The mean of the adaxial and abaxial stomatal conductance measurements was calculated, but due to high variation only the measurements of the abaxial surface were used for analysis. For the stomatal conductance data, outlier values that were greater than +/- 300 mmol m^{-2} s^{-1} from the mean were removed, but no more than two repetitions were removed from any treatment. For chlorophyll fluorescence, the outlier data points that were +/- 0.2 units were removed from the analysis, and no more than 1 repetition removed from any treatment. The outliers were removed prior to analysis and all experiments were analyzed separately. A repeated measures analysis of variance (ANOVA) followed by a Tukey test was used to determine significant differences between treatments at the 5% level. All calculations were done using SAS v. 9.1 (SAS Institute Inc., Cary, NC).

RESULTS

***Phialophora gregata* detection and symptom severity**. Plants infected with type A and B of *P. gregata* in the controlled and field environments tested positive for *P. gregata* with PCR assays (data not shown). Stem symptoms of BSR were observed in all experiments, but foliar symptoms were not observed in all experiments (Table 1). In the greenhouse experiments, foliar symptoms did not develop following inoculation with either type A or B (Table 1). Mean stem symptom severity ratings in the greenhouse were 33% and 43% for the susceptible cultivar infected with type A and B, respectively, and 19% and 13% for the resistant cultivar infected with type A and B, respectively (Table 1). In the growth chamber, foliar and stem symptoms developed in susceptible and resistant plants. Resistant cultivars infected with type A had stem symptom severity of 12% to 19% and foliar severity of 3% to 5%, while plants infected with type B had less than 12% and 4% stem and foliar severity, respectively. Susceptible cultivars infected with type A had stem symptom severity of 47% to 73%, and foliar severity of 18 to 28%, while plants infected with type B had stem severity of 23% to 47% and foliar severity of 5% to 18%, respectively (Table 1). In the field in 2007, the weather was exceptionally hot and dry, and other pathogens killed many plants before BSR symptoms could develop. No foliar symptoms were observed and less than 5% of plants examined had stem symptoms of BSR; thus no data from 2007 is presented. In 2008, the susceptible cultivars infected with type A expressed 12% to 30% stem symptom severity and 27% to 37% foliar severity, and plants infected with type B had 6% to 11% stem severity and less than 9% foliar severity (Table 1). Resistant plants expressed less than 10% foliar and stem severity, regardless of *P. gregata* type (Table 1).

Leaf area. The leaf area of resistant cultivars was not significantly reduced during latent infection. There was, however a non-significant trend for smaller leaf area compared to the control in plants infected with type B during latent infection (Figure 1). The leaf area was reduced in susceptible cultivars for

some inoculation treatments. In the susceptible cultivar, type A reduced leaf area by 30% at 3 and 4 WPI compared to the control, but type B did not reduce leaf area (Figure 1).

As infection progressed to pathogenic, the leaf area of infected resistant and susceptible cultivars decreased compared to controls. The leaf area of resistant plants infected with type B was 30% less than the controls at 8 WPI, approximately the R5 to R6 growth stage (Figure 1); whereas type A did not affect leaf area of resistant cultivars. In the susceptible cultivar infected with type A, the leaf area at 8 WPI was 80% less compared to the control, and type B reduced leaf area of the susceptible cultivar by 30% compared to the controls beginning at 7 WPI (Figure 1).

Stomatal conductance. The stomatal conductance of resistant and susceptible cultivars was not reduced during latent infection by either type of *P. gregata* compared to the control, however, the conductance was reduced during pathogenic infection (Figure 2). In the resistant cultivar, there was a non-significant trend for type A to reduce conductance beginning at 4 WPI, but conductance was significantly reduced by 50% at 7 WPI Conductance of resistant plants infected with type B was 40% less than the control 9 WPI (Figure 2). The conductance of the susceptible cultivar infected with either type A or B began to decline at 5 WPI and continued to the termination of the experiment. At 5 WPI conductance was reduced by 30% when susceptible plants were infected with either type, and by 9 WPI, conductance decreased up to 80% (Figure 2).

Chlorophyll fluorescence. During latent infection in the growth chamber and field, there were no differences in PSII efficiency in resistant or susceptible cultivars infected with type A or B compared to the control (Figure 3 and 4). During pathogenic infection, there were no significant differences between treatments in the growth chamber (Figure 3). In the field, no significant differences in photosynthetic efficiency of infected and uninfected plants were observed during pathogenic infection, but AG2107 and AG2106 infected with

either type A or B of *P. gregata* had a non-significant trend for lower photosynthetic efficiency than control plants (Figure 4). Similarly, for the resistant cultivar AG2106 infected with type B, the photosynthetic efficiency was lower than the non-inoculated plants (Figure 4).

DISCUSSION

Latent infection is a form of parasitism that is common in plants, but is not typically studied until the interaction becomes pathogenic and the host develops symptoms of disease. The primary goal of these investigations was to determine if *P. gregata* modifies the physiology of soybean during the latent infection period. The leaf area, stomatal conductance, and photosynthetic efficiency of resistant and susceptible cultivars were assessed during latent and pathogenic infection. The interaction between soybean and *P. gregata* is an excellent experimental system because *P. gregata* has a latent phase that can last four weeks in controlled environments and eight or more weeks in the field. The interaction then transitions to pathogenic and symptoms of BSR develop. This is the first report of the effects of latent infection by *P. gregata* on soybean physiology and growth. Latent infection is typically thought to be benign, but latent pathogens can potentially parasitize, stress, or weaken plants, and predisposing them to other pathogens or abiotic stress.

Leaf area of soybean was reduced during both latent and pathogenic infection *by P. gregata*, but the extent was dependent on whether plants were resistant or susceptible and if they were inoculated with type A or B of this fungal pathogen. The decrease in leaf area during latent and pathogenic infection is significant for multiple reasons. Small differences in photosynthetic area can result in reduced carbon fixation, growth rate, and resource allocation (5). These reductions could potentially decrease plant defenses and increase susceptibility to pathogens. A second important consideration is that a decline in leaf quantity and leaf area may reduce flower bud formation and yield potential as has been reported for blueberry (13). Although defoliation data

were not collected during latent infection in this current study, defoliation is associated with pathogenic infection by *P. gregata*, and a decrease in leaf number was observed (data not shown). Defoliation during reproductive stages of soybean is associated with decreased yield (2,4). A third factor is that most breeding programs only evaluate cultivars for resistance to type A and not B. Both types reduced leaf area during different stages of infection of resistant and susceptible cultivars.

Stomatal conductance was not altered in resistant or susceptible plants infected with either type of *P. gregaga* compared to the control during latent infection, but was reduced during pathogenic infection. Therefore, *P. gregata* does not appear to disrupt water movement and cause stomata to close during latent infection. This may be partially explained by the observation that although *P. gregata* is a vascular pathogen, less than 5% of vessels are colonized by *P. gregata* during latent infection (A. Impullitti, chapter 2 of dissertation). During pathogenic infection of the susceptible and resistant cultivars, stomatal conductance declined when infected with genotype A or B, which is contrary to a previous report of stomatal conductance increasing in soybean plants infected with *P. gregata* (10). *P. gregata* infects as many as 70% of the vessels of susceptible plants infected with type A during pathogenic infection, which may impede water flow through the xylem vessels and cause stomata to close to prevent water loss (A. Impullitti, chapter 2 of dissertation). Many pathogens produce phytotoxins and/or elicitins that can also disrupt stomatal movement and plant physiology even when symptoms are absent (15). Syringomycin, a phytotoxin produced by *Pseudomonas syringae* pv. *syringae* causes an efflux of K+ in guard cells that results in stomata closure, while elicitins produced by *Phytophthora ramorum* close stomata in the absence of symptoms of sudden oak death (15,17). *P. gregata* produces a group of phytotoxins known as gregatins, which may affect guard cells or cause other effects that lead to stomatal closure (7,8,12).

In this study no differences in photosynthetic efficiency were detected in susceptible or resistant plants infected by type A or B of *P. gregata* during latent or pathogenic infection. Latent infection has resulted in a decrease in quantum yield of PSII in the absence of symptoms of sudden oak death (15). In this experiment, the presence and/or absence of foliar symptom did not appear to influence the results even during pathogenic infection. These results during pathogenic infection were unexpected since gregatins can reduce electron transport during PSII and the cytochrome b/f complex (18). The reduction of electron transport in PSII may not have been enough to reduce the photosynthetic efficiency.

This study reveals how latent and pathogenic infection of soybean by *P. gregata* influences several critical aspects of soybean physiology. Latent infection by *P. gregata* reduced leaf area of the susceptible but not the resistant cultivar. The effect of latent infection on some cultivars may accumulate over time to reduce photosynthetic efficiency causing increased susceptibility to biotic and abiotic stress. Pathogenic infection by *P. gregata* decreased conductance and leaf area, but photosynthetic efficiency was not altered in this study. Furthermore, given that many plants are infected with latent pathogens, the physiological impact and the internal and external cues that trigger transition from latent to the pathogenic phase during plant-fungal interactions merits future investigation.

ACKNOWLEDGEMENTS

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Table 1.1. Foliar and stem symptom severity of susceptible and resistant soybean cultivars infected with type A or B of *Phialophora gregata* or a non-inoculated control in greenhouse, growth chamber, and field environments.

		Greenhouse[a]		Growth Chamber[b]		Field[c]	
Cultivar	***Pg* type**	**Foliar[d]**	**Stem[d]**	**Foliar[d]**	**Stem[d]**	**Foliar[d]**	**Stem[d]**
Susceptible[f]							
AG2107	A	0	33	18	47	27	12
	B	0	43	5	23	9	11
	Control	0		0	0	0	1
Garst 1824	A	na[e]	na	28	73	37	30
	B	na	na	18	47	0	6
	Control	na	na	0	0	0	0
Resistant[f]							
AG2106	A	0	19	5	12	8	6
	B	0	13	2	10	3	3
	Control	0	0	0	0	0	0
IA 2008R	A	na	na	3	19	3	4
	B	na	na	4	12	0	1
	Control	na	na	0	0	0	0

[a] Average of three experiments.
[b] Average of two experiments.
[c] Data from 2008 only.
[d] Mean percent foliar and stem severity of plants at the R5-R6 growth stage in the greenhouse and growth chamber and the R7 growth stage in the field.
[e] Cultivar not evaluated.
[f] Susceptible or genetically resistant to brown stem rot.

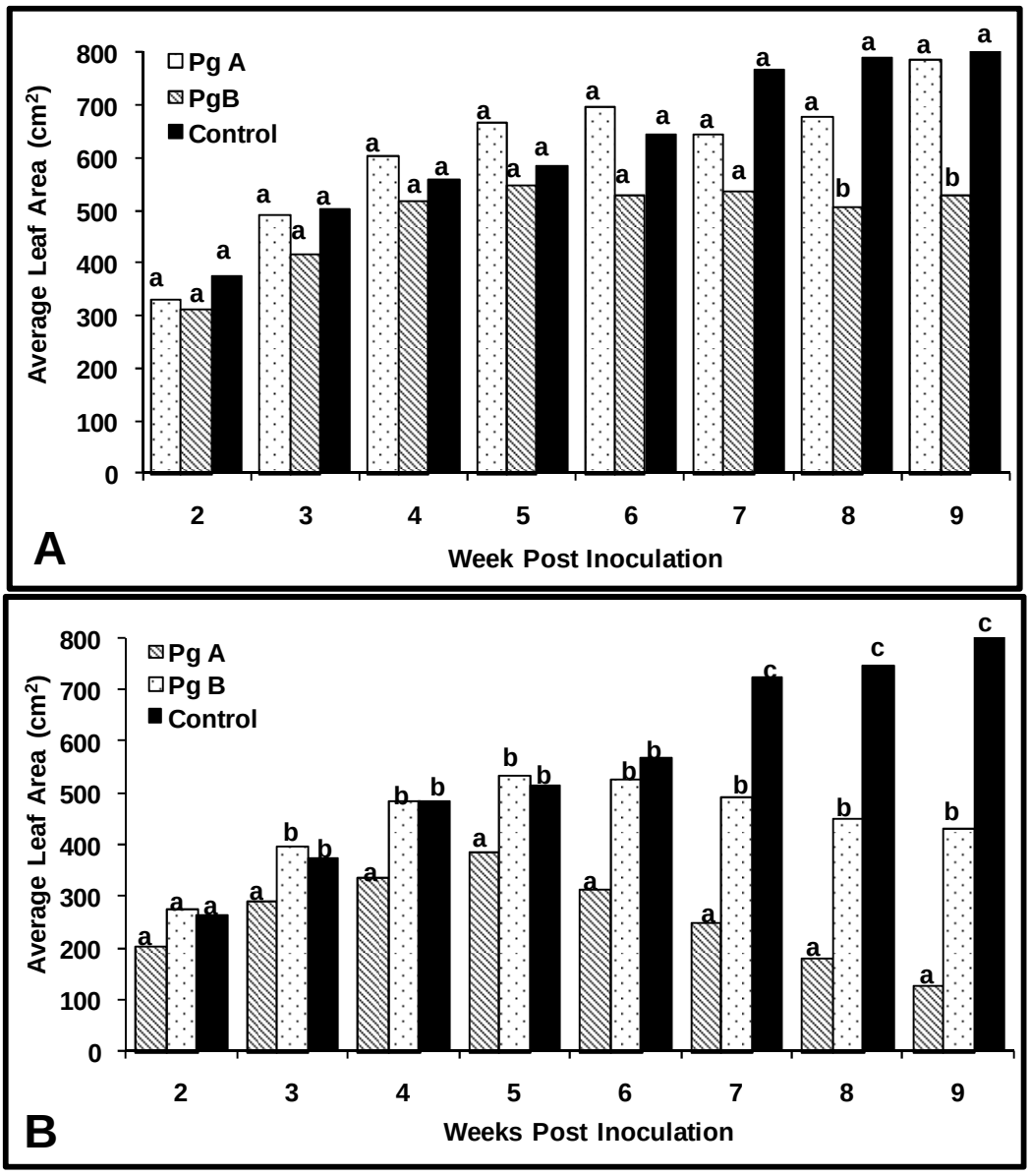

Figure 1.1. The average leaf area of a resistant (**A**) cultivar, AG2106, and a susceptible (**B**) cultivar, AG2107, infected with type A (PgA), B (PgB), or a non-inoculated control as disease progresses from latent infection (1 to 4 weeks post inoculation) to pathogenic infection (5 to 9 weeks post inoculation). Letters indicate whether differences between means of inoculated and non-inoculated plants are significant ($p = 0.05$). Data shown are from one representative experiment, but similar results were observed in two additional experiments.

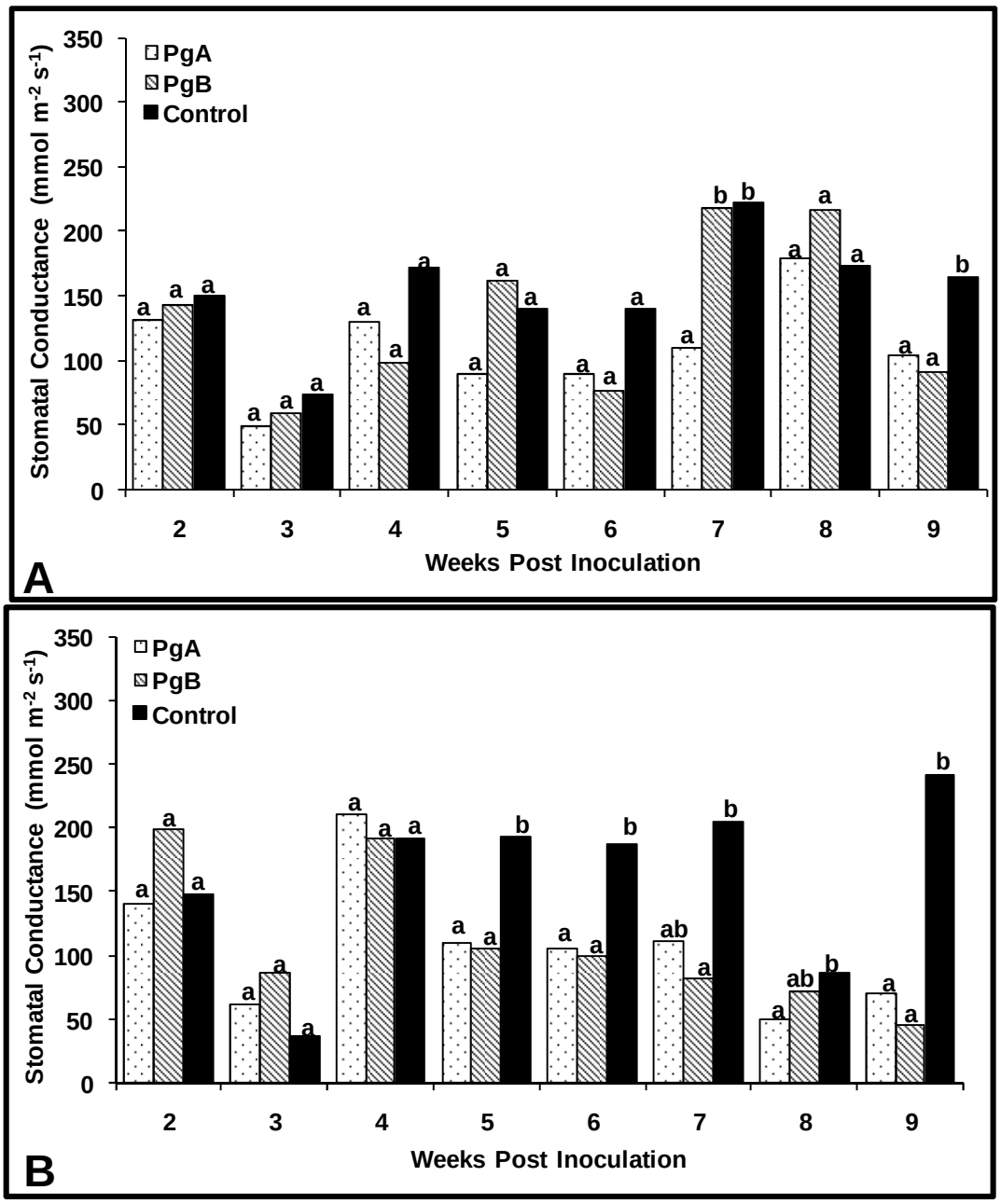

Figure 1.2. The average stomatal conductance of a resistant (**A**) cultivar, AG2106, and a susceptible (**B**) cultivar, AG2107, infected with type A (PgA), B (PgB) or a non-inoculated control as disease progresses from latent infection (1 to 4 weeks post inoculation) to pathogenic infection (5 to 9 weeks post inoculation). Letters indicate whether differences between means of inoculated and non-inoculated plants are significant (p = 0.05). Data shown are from one representative experiment, but similar results were observed in two additional experiments.

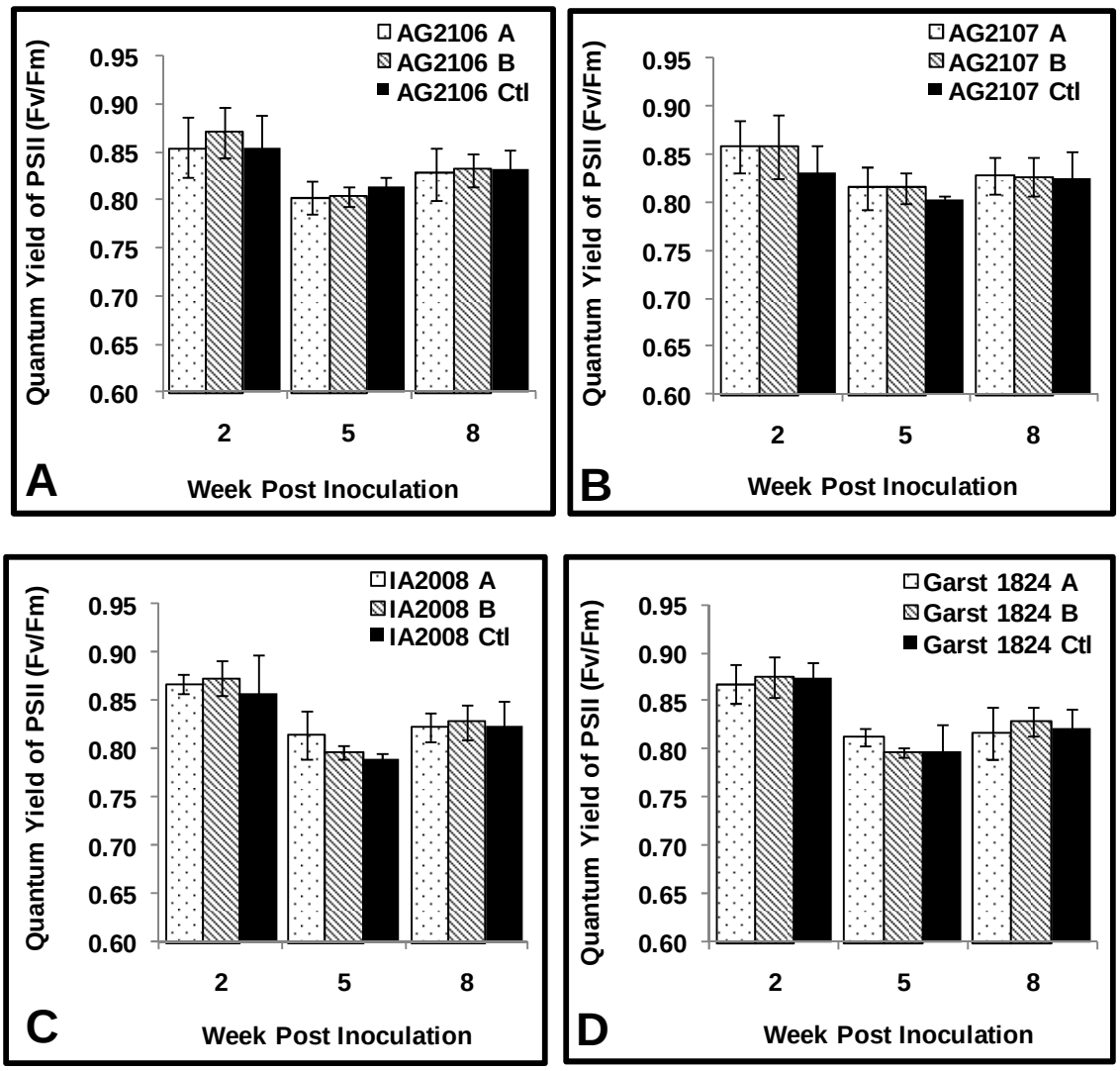

Figure 1.3. Quantum yield of photosystem II (PSII) of dark-adapted resistant AG2106 (**A**) and IA 2008 (**B**) and susceptible AG2107 (**C**) and Garst 1824 (**D**) soybean cultivars during latent (1 to 4 weeks post inoculation) and pathogenic (5 to 8 weeks post inoculation) infection by types A and B of *P. gregata*. Data shown are from one representative experiment from the growth chamber, but similar results were observed in two additional experiments. Each point represents the mean from five replications with three plants per replication, and the error bars represent the standard deviation.

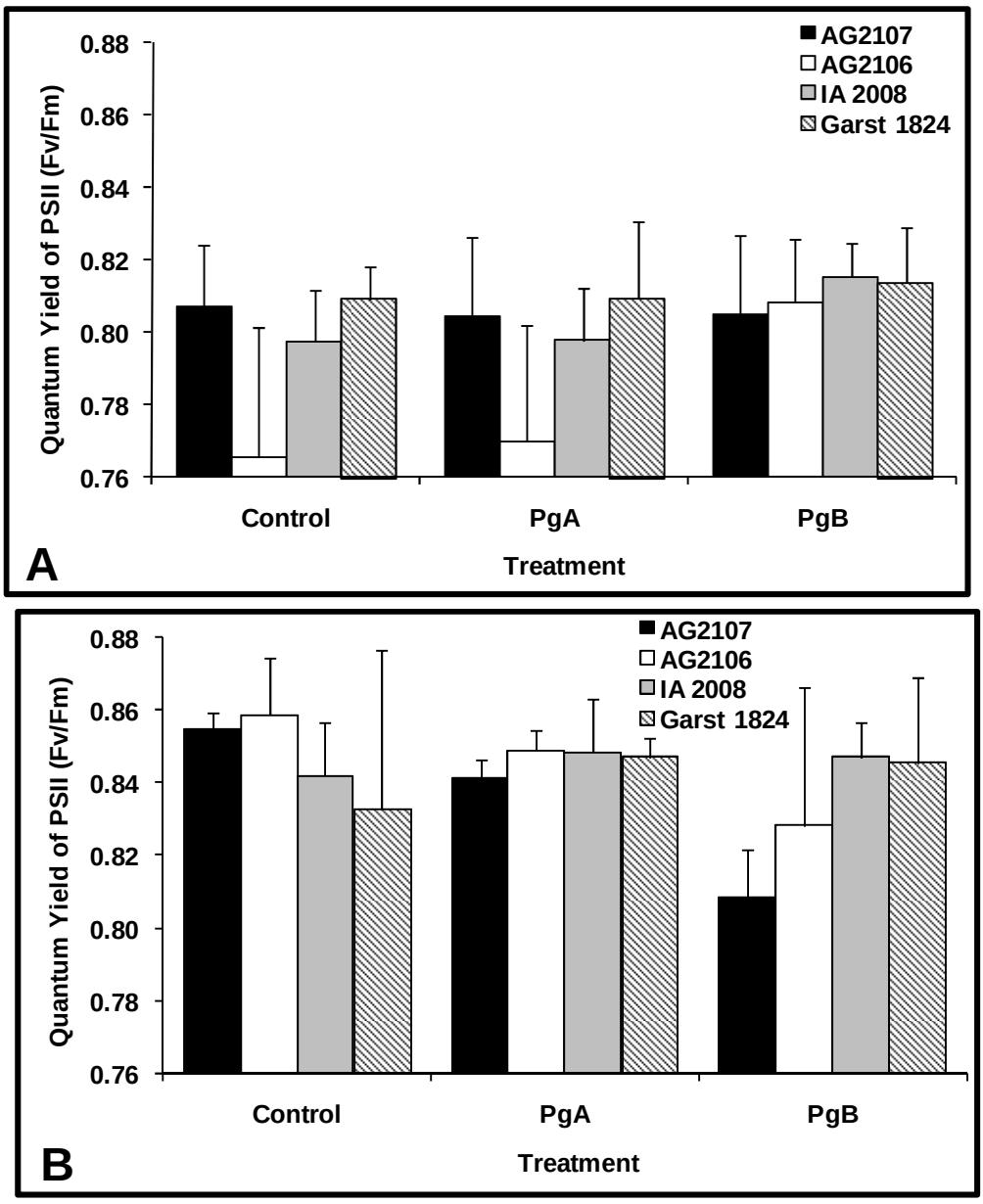

Figure 1.4. Quantum yield of photosystem II (PSII) of dark-adapted resistant AG2106 and IA 2008 and susceptible AG2107 and Garst 1824 soybean cultivars during latent **(A)** (1 to 8 weeks post inoculation) and pathogenic **(B)** (9 to 14 weeks post inoculation) infection by types A and B of *P. gregata* (Pg) in the field. Results from one experiment are shown. Each point represents the mean from four replications with ten plants per replication, and the error bars represent the standard deviation.

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Chapter 2: Use of transmitted light and fluorescent microscopy to investigate the anatomy and colonization of soybean by Type A and B of *Phialophora gregata* during latent and pathogenic infection.

Phialophora gregata colonizes the stems of soybean (*Glycine max*) leading to stem and leaf necrosis and the disease brown stem rot. The interaction between soybean and *P. gregata*, was studied to determine how this pathogen colonizes stems and if fungal colonization influences stem vascular structure during latent and pathogenic phases of infection. Two susceptible cultivars, Corsoy 79 and LN 92-12054, and four resistant cultivars, Bell, IA 2008R, BSR101, and LN 92-12033 were inoculated with an isolate of type A (PgA) that causes foliar and stem symptoms or an isolate of type B (PgB) that causes only stem symptoms. The number of vessels, area of vessels, and percent of vessels colonized by *P. gregata* was determined during latent and pathogenic infection. During latent infection, few differences in the number or area of vessels was observed in resistant or susceptible plants, and *P. gregata* was rarely observed in the plant tissues. As the interaction transitioned to pathogenic, differences were observed in vessel number, but no trends were observed for changes in vessel area. The resistant cultivars Bell, IA 2008R and LN 92-12033 infected with PgA had fewer than 10% of vessels colonized and had 20 to 25% more vessels, while the susceptible cultivar LN 92-12054 had more than 70% of its vessels colonized and 50% fewer vessels compared to the uninfected plants. When infected with PgB, greater than 10% of vessels were colonized and no differences in vessel numbers were observed in Bell or IA 2008 compared to the uninfected plants. In both LN 92-12033 (resistant) and LN 92-12054 (susceptible), 25% of the vessels were infected and the vessel number was reduced by 40% and 30%, respectively. Qualitative differences in fungal colonization were observed during pathogenic infection. *P. gregata* first appeared in the vascular system and then later in the pith as infection progressed. Also, using GFP and RFP isolates, PgB-RFP was observed in the primary xylem, while PgA-GFP was observed exclusively outside of the primary xylem in Bell. The opposite was observed with the susceptible Corsoy 79, where PgA-GFP was observed in the primary xylem and PgB-RFP was limited to the interfascicular region. These results suggest resistant cultivars are able to

produce more vessels and are able to restrict or exclude *P. gregata* from the vascular system. These structural resistance mechanisms potentially compensate for loss of vessel function and prevent disruption of water movement throughout the plant.

INTRODUCTION

Symbiosis between plants and fungi can be mutualistic, pathogenic, or neutral. These interactions are not always distinct, and can sometimes transition between forms. For example, latent infection of plants by pathogens is a common form of parasitism in which the plant and pathogen co-exist for a few days to a few weeks without macroscopic symptoms of disease. Following the latent phase, plants develop symptoms of disease. Latent infection is distinct from endophytic infection, in which endophytes do not parasitize the host and there are no symptoms of disease throughout the association (26). Latent and endophytic infections are cryptic in agricultural crops, and there is limited knowledge on their impact on crop productivity. Pathogens may lead a latent lifestyle if the environment is not favorable for disease development or if the plant is resistant at early growth stages but becomes susceptible as the plant matures.

Numerous economically important fungal pathogens of soybean (*Glycine max* (L.) Merr.) are known to have a latent phase during their disease cycle. Many aspects of the pathogenic phase of these diseases have been investigated, but the latent phase of infection has rarely been studied. The interaction between soybean and the brown stem rot (BSR) pathogen *Phialophora gregata* W.Gams (Allington and Chamberlain) [*Cadophora gregata* Harrington and McNew (12)] is an excellent pathosystem to investigate latent infection due to the long latent phase of the pathogen and the consistency of the transition from the latent to pathogenic phase.

P. gregata is a common soybean pathogen throughout the Midwestern U.S. that infects plants 2 to 3 weeks after planting. Latent infection transition to pathogenic, and symptoms of disease are observed during soybean reproductive growth stages, approximately 10 to 12 weeks after planting when there are cool night temperatures, short day length, and plants begin to mature (22,28). Typical symptoms of BSR include leaf abscission, interveinal chlorosis

and necrosis of leaves, and internal browning of vascular and pith tissues in the stem (1). Symptoms of BSR differ depending on whether plants are infected with type A or B of the pathogen. Type A of *P. gregata* causes foliar and internal stem symptoms, and type B causes stem symptoms, but foliar symptom severity is typically less than 10% (11,14). Although the symptomatic differences and the association of resistant and susceptible cultivars with type A or B are clearly documented, many questions remain about how *P. gregata* colonizes plants during latent and pathogenic infection (6,14,18).

Infection and colonization of plant tissues during pathogenic infection by pathogens is distinct from endophytic and latent infection (4,13,26,38). For example, *Fusarium verticilliodes* commonly infects corn and is often referred to as an endophyte, latent pathogen, or pathogen depending on the environment, water availability, and genetics of the host and fungus (4,8,15,24,38). During asymptomatic infection, hyphae of *F. verticilliodes* are limited to intercellular colonization, but during symptomatic infection, hyphae colonize stem tissues inter- and intracellularly, and stalk height, girth, and biomass is reduced (4,24,38). In another example, *Colletotrichum acutatum* was used to investigate pathogenic colonization of strawberry and endophytic colonization of solanaceous hosts (13). On strawberry, appresoria penetrate host tissue, hyphae colonize intracellularly, and cause necrosis. On endophytic hosts, appresoria of *C. acutatum* fail to germinate or the fungus has an epiphytic lifestyle, and hyphae are restricted to intercellular growth (13). These microscopic investigations have revealed differences in localized infection and growth of fungi, but have not provided a clear depiction of the anatomical modifications or colonization of resistant and susceptible hosts through the disease cycle.

The goal of this study was to determine if differences in soybean stem colonization and anatomy occur during latent and pathogenic infection by *P. gregata*, and to learn how the two types of this pathogen colonize and interact *in planta*. Transmitted light microscopy was used for some of the studies, to

study anatomical differences, but cannot be used to distinguish types A and B and other fungi that colonize plant tissues. Therefore, type A and B were modified to express green or red fluorescent protein (GFP/RFP). These fluorescent proteins are strongly expressed with the proper promoters in fungi, and have provided valuable insight on gene expression, protein localization and colonization of microbes in plants (2,5,13,17,24,37). The objectives of this study were to: 1) Compare the vascular anatomy of stems of resistant and susceptible soybean cultivars infected with types A and B of *P. gregata*; 2) Determine if there are differences in colonization of resistant and susceptible soybean stems during latent and pathogenic infection by types A and B of *P. gregata*; 3) Determine if simultaneous inoculation of types A and B results in concurrent colonization of the same plant tissues.

MATERIALS AND METHODS

Isolate descriptions and transformation. The type A isolate (OH2-3) of *P. gregata,* transformed to express GFP (PgA-GFP), and the transformation procedure were supplied by C. Bronson at Iowa State University. The type B isolate (MNRF-ss1) was transformed to express RFP using the same procedure as follows. A single spore isolate was grown on green bean agar (GBA) for 4 to 5 weeks to promote sporulation (31). Conidia were collected by pouring 5 to 10 ml of sterile green bean broth onto each plate and scraping with a sterile spatula (31). The spore suspension was incubated in the dark at 25℃ for 48 hrs to promote germination of spores. Protoplasts were prepared from the germinating conidia using a modified enzyme-osmoticum solution that contained 7.5 mg/ml driselase, 100 µg/ml chitinase, and 7.5 mg/ml Glucanex (Sigma-Aldrich, St. Louis, MO) instead of Novozyme 234 (33,34). The RFP vector, pCA56, supplied by L. Ciuffetti at Oregon State University, contains a modified version of the hygromycin resistance gene *hph* and an optimized version of DsRed fluorescent protein driven by the *ToxA* promoter (2,33,34,35). The vector was linearized with *Sac*I prior to a polyethylene glycol (PEG)

transformation (2,5,17). After transformation, the treated protoplasts were mixed into a cell wall regeneration medium and 10 to 15 ml of the mixture was poured into a petri plate (34). The cell wall was allowed to regenerate for 24 hrs at ambient temperature and lighting, and then the regeneration plates were overlaid with 1% water agar containing 150 µg/ml hygromycin B and incubated for 10 to 14 days at 25 C. Potential transformants were transferred to GBA containing 100 µg/ml hygromycin B.

Evaluation of transformants. Insertion of RFP into Type B was tested by PCR with the forward primer U32 and the reverse primer U33 and expression of RFP was tested by fluorescent microscopy (9). Confirmed RFP isolates were stored in sterile water at 14℃. Two RFP transformants with similar morphology to the wild-type (WT) were selected for comparison of the radial growth, dry mass, and sporulation of the WT and RFP transformants. Radial growth was assessed by placing a 5 mm diameter disk of an actively growing culture on green bean agar (GBA), incubating at 25℃ with a 12 hr photoperiod, and then measuring the diameter of the colony after 3 weeks. Dry mass and sporulation were assessed by growing the WT and transgenic isolates in flasks of soybean seed broth (SSB) (12 g of BSR susceptible soybeans/100 ml of water) that were shaken daily and incubated at room temperature (approximately 23℃) with ambient fluorescent light. After 21 days, conidia were counted using a hemacytometer and the remaining broth cultures were filtered through 4 layers of cheese cloth to collect mycelium. Mycelium was placed in a drying oven at 35℃ for 12 hrs and weighed.

Virulence and pathogenicity of transformed and WT isolates were assessed in three growth chamber experiments. Soybean cultivars Bell and Corsoy 79 were inoculated with the WT of type A or B, type A-GFP (PgA-GFP), type B-RFP (PgB-RFP), or were non-inoculated. Inoculum was produced by growing isolates in SSB for 21 days. Plants were grown in coarse-grade vermiculite to the VC-V1 growth stage and inoculated by dipping wounded roots into an inoculum suspension containing 6.0 x 10^6 cfu/ml of *P. gregata* (14).

Three inoculated plants per treatment were transplanted into a 6 inch pot containing Sunshine LP5 (Sun Gro Horticulture, Bellevue, WA). Inoculated seedlings were grown in a growth chamber with a 12 hr photoperiod at a 23℃ day and 20℃ night temperature. Foliar and stem symptoms were assessed 7 weeks post-inoculation. Percent foliar severity was determined by dividing the number of healthy trifoliates by the symptomatic trifoliates and multiplying by 100. Stems were split longitudinally to assess the percent of the stem length that was necrotic.

Evaluation of infection phases and anatomical differences. Experiments 1 and 2 were conducted using the susceptible cultivar Corsoy 79 and the resistant cultivar, Bell (36). A third experiment used the resistant cultivars, IA2008R, BSR101, and the near isogenic resistant line LN 92-12033 (*Rbs2*) and its corresponding susceptible line LN 92-12054 (23,36). Plants were inoculated with the WT isolates and grown in the growth chamber as previously described. During experiments 1 and 2, three plants from each treatment were selected for analysis at 1, 2, 4, 6, and 8 weeks post- inoculation (WPI). During experiment 3, four plants from each treatment were selected at 2 and 6 WPI.

To section plant material, 0.5 to 1.5 cm segments of stem were taken from the base, middle, and apex of each plant and embedded in 6% water agar. A Vibratome 1000 Plus Sectioning System (Vibratome Co., St. Louis, MO) was used to slice 5 to 20 µm thick cross-sections (XS). Longitudinal sections (LS) of stems were collected at 2 WPI, and root XS and LS were taken at 2 and 8 WPI. Sections were mounted on a microscope slide, stained with lactophenol aniline blue (Remel, Lenexa, KS), washed with water, and examined with an Eclipse e600 Nikon Microscope (Nikon Instruments Inc., Melville, NY). Transmitted light microscopy (TLM) images were captured using a Nikon DXMF 1200 camera with the software ACT1 (Nikon Instruments Inc., Melville, NY). A composite picture of the stem XS was taken at 40x. The magnification was then increased to 200x, and successive images of the entire stem XS were captured for analysis. The image from the middle section of each stem was used to

determine the number of vessels, area of vessels, and percent of vessels colonized with *P. gregata* with the aid of the software Image J, from the National Institutes of Health (27). The middle cross section of plants during weeks 1, 2, 4, 6, and 8 were analyzed during experiment 1 only. Based on the data collected during experiment 1, data during experiments 2 and 3 was only collected at 2 and 6 WPI for analysis of latent and pathogenic infection, respectively. Complete XS segments of the base and apex were difficult to obtain due to woody, secondary growth and immature tissue development, respectively, and therefore these sections were only used to determine the presence or absence of *P. gregata*. A total of 55,113 vessels (n = 168 middle stem XS) were analyzed from the transmitted light experiments. The stem sections adjacent to the area used for analysis of XS were collected during each experiment, chopped into 1 to 2 mm pieces and then stored at -20℃. DNA was extracted and used to verify infection by *P. gregata* using PCR (19). Standard PCR (sPCR) was initially used for detection, but a more sensitive real-time quantitative PCR (qPCR) assay was used if inoculated samples were negative using sPCR (20).

Assessment of colonization by types A and B. The cultivars Bell and Corsoy 79 were grown to the V1 growth stage and inoculated as described with isolates PgA-GFP and PgB-RFP separately and also co-inoculated with a 1:1 mixture of both isolates. Inoculated and non-inoculated plants were grown, symptoms assessed, and plant tissues embedded and sectioned as described. Sections were placed on a slide with a droplet of water and covered with a cover slip.

A Nikon C1si laser scanning confocal microscope (LSCM) (Nikon Instruments Inc., Melville, NY) at the University of Minnesota Imaging Center was used for fluorescent microscopy. This instrument is capable of true spectral imaging, and the autofluorescence of the plant tissues and the fluorescence of GFP and RFP can be unmixed. GFP images were acquired by excitation with a 488 argon laser at 12% intensity in standby modus, and the RFP was excited at

561 nm at 15% intensity, with the fluorescence being detected with 32-channel photo multiplier tubes (PMTs) at a range of 505 to 680 nm which were corrected for differential wavelength sensitivity. A spectral gain of 160 and pixel dwell speed of 6 µs was used. The images presented are projections of a z-series (maximum intensity projection images) collected at 0.5 micron steps. The z-series merger and unmixing of autofluorescence and GFP and/or RFP fluorescence was done using spectra from known GFP and RFP (Clontech, Mountain View, CA) and by selecting regions of auto-fluorescence in control plants and using the Nikon EZ-C1 Viewer, ver. 3.60.

Statistical analysis. Statistical analyses were conducted using the Proc GLM procedure in SAS (SAS v9.1 Institute Inc., Cary, NC). The data for the two experiments that evaluated transformants were combined because there were no significant differences between experiments, analyzed using ANOVA, and means were compared using a LSD (P = 0.05). Data from the transmitted light experiments that assessed the quantity of vessels and average area of vessels were analyzed separately by an ANOVA. If the *F* test was significant (P = 0.05) means were compared using a Tukey test.

RESULTS

Transformation. Types A and B of *P. gregata* were transformed to express GFP and RFP, respectively. The PgA-GFP isolate was similar to the WT strain based on colony growth and mycelial dry mass, but its sporulation was compromised by 25 to 50% compared to the WT (data not shown). The reduction in sporulation did not affect stem symptoms produced in the susceptible cultivar Corsoy 79 (data not shown). Six isolates of type B that expressed RFP and were hygromycin resistant were obtained in this study. Of these transformants, two were selected for comparison with the WT isolate based on their phenotype and growth. The phenotype was modified and fitness reduced of one transformant, MNRF-RFP2 compared to PgB-RFP and the WT (**Figure 2.1**). The phenotype of the transformed isolate PgB-RFP and the WT

isolate were similar (**Figure 2.1**). PgB-RFP had a radial growth rate, dry mycelial mass, and sporulation that were statistically the same as the WT isolate (Table 2.2). Likewise, the stem symptom severity of the resistant cultivar Bell, and the susceptible cultivar, Corsoy 79, were similar when inoculated with either the WT or PgB-RFP isolate (Table 2.2). As expected, few to no foliar symptoms were observed in soybeans following inoculation with this isolate.

The expression of RFP in isolate PgB-RFP was confirmed with fluorescent microscopy and the insertion of RFP gene was confirmed using PCR (9). Transformants expressing RFP also had a pink phenotype when grown on 1/2x PDA when compared to the WT (**Figure 2.1**). The expression of RFP was stable, and bright throughout the experiments using PgB-RFP. No loss of fluorescence or photo bleaching after LSCM was observed.

Symptoms and *Phialophora gregata* detection. *P. gregata* was never detected from non-inoculated plants using sPCR or qPCR, nor were symptoms of infection observed. Detection of *P. gregata* in inoculated plants varied depending on the cultivar and the WPI (Table 2.3). At 2 WPI no symptoms were observed, but several plants tested positive for PgA or PgB using sPCR or qPCR. Microscopic analysis of XS revealed that less than 10% of vessels were infected for all treatments and cultivars (Table 2.3). At 6 WPI symptoms were observed to varying degrees in all inoculated plants, and all plants tested positive for *P. gregata* except for two samples of IA 2008 and one sample of LN 92-12033 infected with PgA. These two samples had less than 1% of their vessels infected and are resistant to *P. gregata* (Table 2.3). Fungal hyphae were present in less than 1% of vessels of non-inoculated plants 2 and 6 WPI. There was a strong, positive correlation between stem severity and the percent of vessels infected ($R^2 = 0.72$) (data not shown).

Comparison of vessel number and area. We have classified 1 to 4 WPI as latent infection and 5 to 8 WPI as pathogenic infection based on timing of macroscopic symptom development in controlled environments. Of the two separate experiments conducted using Bell and Corsoy 79, only data from

experiment 1 is presented. Similar trends were seen for experiment 2, unless noted otherwise.

During latent infection, stem anatomical features of infected and non-infected plants were typical of normal soybean anatomy (16). A diagram of the anatomy of a soybean stem is included for reference (**Error! Reference source not found.Error! Reference source not found.**Figure 2). Few xylem vessels were infected and differences in the number of vessels in infected and non-infected plants were not evident 2 WPI (Figure 3A). The area of xylem vessels of most resistant cultivars was not affected by *P. gregata* infection at 2 WPI, but the average area of vessels was different in infected and non-infected susceptible cultivars (Figure 4A). The susceptible cultivars, Corsoy 79 and LN92-12054, had 20 to 30% less vessel area when infected with PgB or PgA, respectively, compared to the uninfected plants (Figure 4A).

During pathogenic infection with PgA, differences in the number of vessels in infected plants compared to non-infected plants were observed. The resistant cultivars Bell, IA2008, and LN 92-13033 had 20 to 25% more vessels compared to the uninfected plants (Figure 3B). The susceptible cultivar LN 92-12054 when inoculated with PgA had 50% fewer vessels compared to the uninfected plants and the resistant near isogenic line LN 92-12033 (Figure 3B). The number of vessels in resistant cultivar BSR101 was similar to LN 92-12054 when infected with PgA and had fewer vessels than the uninfected plants. No significant differences in vessel quantity were seen between the susceptible cultivar Corsoy 79 infected with PgA compared to the uninfected plants, but there was a trend of fewer vessels in the infected plants compared to the uninfected plants during the two separate experiments.

Differences in the number of vessels in plants infected with PgB compared to non-infected plants were observed (Figure 3B). Vessel numbers were significantly reduced by 40% and 30% in LN 92-12033 and LN 92-12054, respectively, when infected with PgB compared to the uninfected plants (Figure 3B). The cultivar BSR101 had significantly fewer vessels when infected with

PgB compared to the uninfected plants (Figure 3B). No significant differences were observed in the number of vessels of Bell, IA2008, or Corsoy 79 infected with PgB compared to the uninfected plants (Figure 3B).

Average vessel areas were generally smaller in susceptible cultivars than in resistant cultivars infected with either PgA or B compared to the uninfected plants at 6 WPI. Corsoy 79 and LN 92-12054 had a 25% smaller vessel area when infected with either PgA or PgB compared to the uninfected plants (Figure 4B). The vessel area of resistant plants varied by cultivar, and no consistent trend among all the resistant cultivars was observed. The resistant cultivar IA 2008 was similar to the susceptible cultivars in that the vessel area of infected plants was 20% smaller compared to the uninfected plants. There were no significant differences between the treatments in the resistant cultivars Bell or LN 92-12033, but a slight trend was observed for these cultivars infected with PgB to have a smaller vessel area than the uninfected plants or plants infected with PgA (Figure 4B). In contrast, plants of the resistant cultivar, BSR101, infected with PgA had a 20% greater vessel area than the uninfected plants (Figure 4B).

Depiction of latent colonization. During 1 to 4 WPI, no foliar symptoms or vascular discoloration in the stems of Bell or Corsoy 79 was apparent (Figure 5). At 1 WPI, *P. gregata* was not detected using sPCR or qPCR (data not shown) and no fungal structures were observed in the root, base, middle, or apex sections of plants infected with either the WT or fluorescent isolates (Figure 6). At 2 WPI, *P. gregata* was detected in all plants using sPCR or qPCR (Table 2), and microscopic necrosis was observed in the vascular system of the root of Corsoy 79 infected with PgB (Figure 7). At 2 and 3 WPI, less than 10% of xylem vessels in the stems of most plants were colonized by fungal hyphae, but these observations using TLM were challenging because the amount of fungal material was minimal making it difficult to determine if it was really *P. gregata* or if it was an artifact from staining. Fungal structures were seen in the xylem vessels and in interfascicular areas using fluorescent imaging at 3 WPI

(Figure 10). Longitudinal sections from the middle did not provide any more information on the location of *P. gregata* during 1 to 3 WPI using TLM or LSCM. At 4 WPI, types A and B of *P. gregata* were observed in the base, middle, and apex of resistant and susceptible plants using TLM, but neither type A or type B was observed in the pith (parenchyma) or cortex of stems (data not shown).

Depiction of pathogenic colonization. Microscopic observations of pathogenic infection, 5 to 8 WPI, were similar in susceptible and resistant plants, except that infection was more severe in susceptible plants. At 6 and 8 WPI, *P. gregata* was detected in susceptible and resistant cultivars 100% of the time using one of the PCR methods, and foliar and stem symptoms were observed (Table 2, Figure 8). The fungus colonized the xylem vessels in the middle and apex of resistant and susceptible cultivars with some vessels being 100% colonized by *P. gregata*, while others had severe browning, but no fungal hyphae inside the vessel (Figure 8). At 8 WPI, hyphae were first observed moving into the adjacent parenchyma cells of the pith, and conidia were also observed in some vessels.

The tissue structure of infected plants differed from the uninfected plants. At 8 WPI, plants infected with *P. gregata* had the same anatomical features present, but to different extents. The width of the cylindrical ring of secondary xylem became smaller and less pronounced in infected plants compared to uninfected plants of Corsoy 79 (Figure 9). A similar trend, but to a lesser extent was observed in Bell. The secondary xylem of Bell was similar in plants infected with PgA and the uninfected plants (Figure 9). Bell infected with PgB had less secondary xylem than the uninfected plants. Xylem vessels collapsed, the cambium layer began to breakdown, and vessel occlusions were also observed, but not to a great extent at 8 WPI when inoculated with either type A or B (Figure 9).

Individual and co-infection of PgA-GFP and PgB-RFP. When PgA-GPF or PgB-RFP were separately inoculated in Bell or Corsoy 79, results were similar to those seen using the WT isolates and TLM. Neither type was

observed in XS or LS during latent infection. As the interaction transitioned to pathogenic, PgA-GPF and PgB-RFP colonized the primary vessels, pith, and interfascicular region. When the two types were used to co-inoculate plants, no differences were observed during latent infection, but differences in the colonization pattern for each type was observed during pathogenic infection. Co-inoculation of Bell resulted in PgB-RFP being readily observed in the vessels, while PgA-GFP was limited to the interfascicular area outside of the primary and secondary vessel elements (Figure 10). There were a few rare instances when both PgA-GFP and PgB-RFP could be observed in the same vessel (Figure 10). The different colonization patterns were observed in the susceptible cultivar. PgA-GFP readily colonized the primary vessels, while PgB-RFP was limited to the interfascicular region (Figure 10). PgA-GFP and PgB-RFP were not seen co-infecting a vessel in the susceptible cultivar.

DISCUSSION

Many soybean pathogens are latent for an extended period of time, however, this stage of infection is rarely investigated (29). The purpose of this study was to investigate latent and pathogenic colonization of soybean stems by types A and B. Our results are the first to identify differences in the vascular structure of resistant and susceptible cultivars during latent and pathogenic infection. These results provides insight into the mechanism of BSR resistance and active responses to infection by *P. gregata.,* as well as qualitative differences in colonization by the A and B types and how they interact when infecting the same plant.

The PgA and PgB wild-type (WT) isolates were used to assess differences in internal stem anatomy of resistant and susceptible soybean cultivars during latent and pathogenic infection. During latent infection, neither the vessel quantity nor the average area of vessels was altered in resistant plants following infection with type A or B compared to the uninfected plants. In susceptible plants, the number of vessels was not affected, but the average

area of vessels was reduced by 10 to 50% compared to non-infected plants. As the interaction transitioned from latent to pathogenic, three main differences developed in the vascular system of resistant and susceptible plants. First, the cultivars Bell, IA 2008, and LN 92-12033, which are all resistant to type A of *P. gregata,* had 20 to 25% more vessels than the uninfected plants. Second, the cambium layer, which was reduced in susceptible plants infected with *P. gregata* compared to the uninfected plants, was reduced to a lesser extent in infected resistant plants compared to uninfected. Third, fewer than 10% of vessels of the resistant cultivars, Bell, IA 2008, and LN 92-12033 were infected with type A, compared to greater than 50% of vessels of the susceptible cultivar LN 92-12054. The vessel area of plants was not an ideal method to separate resistant from susceptible cultivars, as the area appeared to be more dependent on cultivar genotype rather than on resistance or susceptibility.

These results suggest different mechanisms of resistance to *P. gregata*. One proposed mechanism is the regeneration of the vascular cambium and vascular system following infection. The increase in vessel number and the unaltered cambium layer suggests resistant plants infected with type A may produce vessels to compensate for loss of vessel function and disrupted water movement through the plant due to vessel colonization. All cultivars used in this study were considered to be susceptible to type B as most breeding programs only evaluate for resistance to type A. Susceptible plants infected with type B had less secondary growth and a smaller cambium layer indicating the vascular tissues were unable to regenerate in response to infection by *P. gregata*. Similarly, reduced vessel number, diameter, and vascular cambium were observed in tomato (*Lycopersicon esculentum*) infected with *Fusarium oxysporum* f. sp. *lycopersici* compared to the uninfected plants (7). Another potential resistance mechanism is the ability of resistant plants to restrict or exclude the pathogen from the vascular system. Less than 10% of vessels of the resistant cultivars, Bell, IA2008, and LN 92-12033 were infected with type A, compared to greater than 50% of vessels of the susceptible cultivar LN 92-

12054. These results are similar to other plant-pathogen systems during pathogenic colonization in that resistant cultivars are able to restrict pathogen growth (25,37).

Latent and pathogenic infection were distinguished based on timing and observed differences in colonization. During latent infection, fungal colonization was rarely observed in any plant tissues using either transmitted or fluorescent light microscopy, but were able to detect the fungus in some, but not all stems of plants using sPCR and/or qPCR at 1 or 2 WPI. We hypothesize there were no more than trace amounts of *P. gregata* within the stem at this time, thus making it difficult to visualize within the plants. *P. gregata* may also have been surviving intercellularly as do many other asymptomatic fungal colonizers (21,24,38). Another hypothetical explanation is that *P. gregata* was limited to the roots during latent infection. Other studies in different plant-pathogen systems have shown some vascular pathogens are limited to the root due to vascular occlusions (3,32,37). However, from our limited observations of roots, we do not think this was the case since *P. gregata* was rarely observed in the roots. We are confident that *P. gregata* was in the stems based on our PCR results and because *P. gregata* can be isolated from the apex of infected plants one hour post-inoculation, suggesting conidia move throughout the vascular system and systemically colonize the plant during latent infection (30,31). A single conidium is 3.4 x 7.6 µm which from our observations is smaller than the average soybean vessel. From our molecular methods, we concur with Tabor et al. (2007) that the fungus is capable of moving to the apex of plants during the early stages of infection, but the amount of fungal material is minimal.

During pathogenic infection, *P. gregata* was readily observed in plant stems using both TLM and fluorescent LSCM. By 4 WPI, type A and type B were present in the vascular system of stems in resistant and susceptible cultivars and vascular browning was observed microscopically, but macroscopic symptoms were not observed. By 6 WPI, macroscopic symptoms began to be observed in the stem and on the leaves, and it became evident that the two

pathogen types had begun to colonize the primary vessels of the vascular system. Fungal hyphae were observed in areas with and without evidence of stem browning. Vascular colonization was required before stem browning and before colonization of the pith tissues were observed.

Transformation of the type A and B of *P. gregata* to express GFP and RFP was successful. The PgA-GFP isolate was similar in growth and pathogenicity to the WT isolate, but had slightly less sporulation, suggesting the GFP insertion site or expression of GFP may have decreased its fitness. The PgB-RFP isolate behaved like the WT isolate in all aspects tested. The fluorescent isolates confirmed the observations of the WT isolates using TLM and also offered novel insight into differences in pathogenic colonization of soybean by the PgA-GFP and PgB-RFP isolates. PgA-GFP infected vessels of Corsoy 79, but PgB-RFP was limited to the interfascicular areas during pathogenic infection of co-inoculated plants. Corsoy 79 is susceptible to both types so it is interesting that PgA is a better colonizer of the vascular system than PgB when the two types are co-inoculated. The xylem is an ideal microhabitat for many pathogens due to the delivery of water and solutes from the plant roots to the leaves, and the ability of fungal spores to be transported systemically throughout the plant. In Bell, which is susceptible to PgB and resistant to PgA, PgA-GFP was limited to the interfascicular region while PgB-RFP was observed in the primary vessels (6,18).. Therefore, our results suggest susceptibility to *P. gregata* is a result of the pathogen colonizing the vascular system, while resistant plants are able to exclude it to the interfascicular region. The anatomical analysis also indicated more vessels were infected by type B in Bell than type A, while in Corsoy 79, more vessels were infected by type A than B.

Latent infection is a well known but poorly understood phenomenon that is often considered one of the highest forms of parasitism since plant and pathogen can co-exist, in some cases, for long periods of time. Now we have a better understanding of colonization during latent and pathogenic infection by

studying the interaction between soybean and *P. gregata*. Many questions remain on differences in latent and pathogenic infection in this pathosystem. It is unknown what initiates the pathogen to move into the vascular system, and how resistant plants restrict colonization of vessels. Also, in co-infected plants, PgA-GFP readily colonized the vessels while PgB-RFP was limited to the interfascular region. Future studies should investigate if type A is more aggressive or is a better competitor and grows faster than type B and if latent infection has a significant negative impact on the host.

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Table 2.2. Comparison of growth and virulence of wild-type and transformed isolates of *Phialophora gregata* Type B.

Isolate	Colony Diameter (cm)[a]	Dry Mass (mg)[b]	Conidia/ml[b]	Stem Severity (%)[c]	
				Bell[d]	Corsoy 79[e]
MNRF-ss1[f]	3.43	31.83	9.6×10^5	16	30
MNRF-ss1-RFP[g]	3.62	30.28	8.2×10^5	13	24
MNRF-ss1-RFP2	1.32	24.33	6.8×10^4	0	5
P value	<0.01	<0.01	<0.01	<0.01	<0.01
LSD (p = 0.05)	0.72	5.46	6.5×10^5	6	6

[a] Measurements taken after 21 days of growth on green bean agar.
[b] Measurements taken after 21 days of growth in soybean seed broth.
[c] Stem ratings are the mean of six stems 7 weeks after inoculation.
[d] Resistant cultivar.
[e] Susceptible cultivar.
[f] Wild-type (WT).
[g] Red Fluorescent Protein (RFP).

Table 2.3. Detection of *Phialophora gregata* in stems with PCR, observation of hyphae in vessels, and stem symptom severity of resistant and susceptible cultivars at 2 and 6 weeks post inoculation.

	2 Weeks Post Inoculation			6 Weeks Post Inoculation		
Cultivar and Inoculation	**Detection of *P. gregata* (%)[a]**	**Vessels Infected (%)[b]**	**Stem Severity (%)**	**Detection of *P. gregata* (%)**	**Vessels Infected (%)**	**Stem Severity (%)**
Bell (R)[c]						
Expt 1						
A[d]	100	6.2	0	100	6.9	13
B[e]	33	6.1	0	100	13.9	2
Control	0	1.2	0	0	0.0	0
Expt. 2						
A	100	1.1	0	100	3.0	10
B	33	1.6	0	100	29.9	2
Control	0	0.0	0	0	0.0	0
BSR101 (R)						
A	75	3.3	0	100	79.8	48
B	0	0.4	0	100	13.6	23
Control	0	0.0	0	0	0.0	0
Corsoy 79 (S)						
Expt. 1						
A	0	8.8	0	100	49.2	45
B	100	3.2	0	100	17.8	12
Control	0	0.7	0	0	0.4	0
Expt. 2						
A	100	1.3	0	100	65.4	73
B	33	3.2	0	100	14.1	7
Control	0	0.2	0	0	0.0	0
IA 2008 (R)						
A	33	1.3	0	50	0.8	10
B	0	0.6	0	100	5.7	23
Control	0	0.1	0	0	0.0	0
LN 92-12033 (R)						
A	0	0.0	0	75	7.9	6
B	50	0.1	0	100	27.7	50
Control	0	0.0	0	0	0.0	0
LN 92-12054 (S)						
A	75	0.0	0	100	73.3	50
B	100	0.0	0	100	25.2	60
Control	0	0.0	0	0	0.0	0

[a] Detection of *P. gregata* in plants. If *P. gregata* was not detected using standard PCR, a more sensitive real-time quantitative PCR assay was used.
[b] The percent of vessels infected with hyphae in a cross section.
[c] Reaction of soybean cultivars to *P. gregata*; R = resistant, S = susceptible.
[d] Plants infected with Type A of *P. gregata*.
[e] Plants infected with Type B of *P. gregata*.

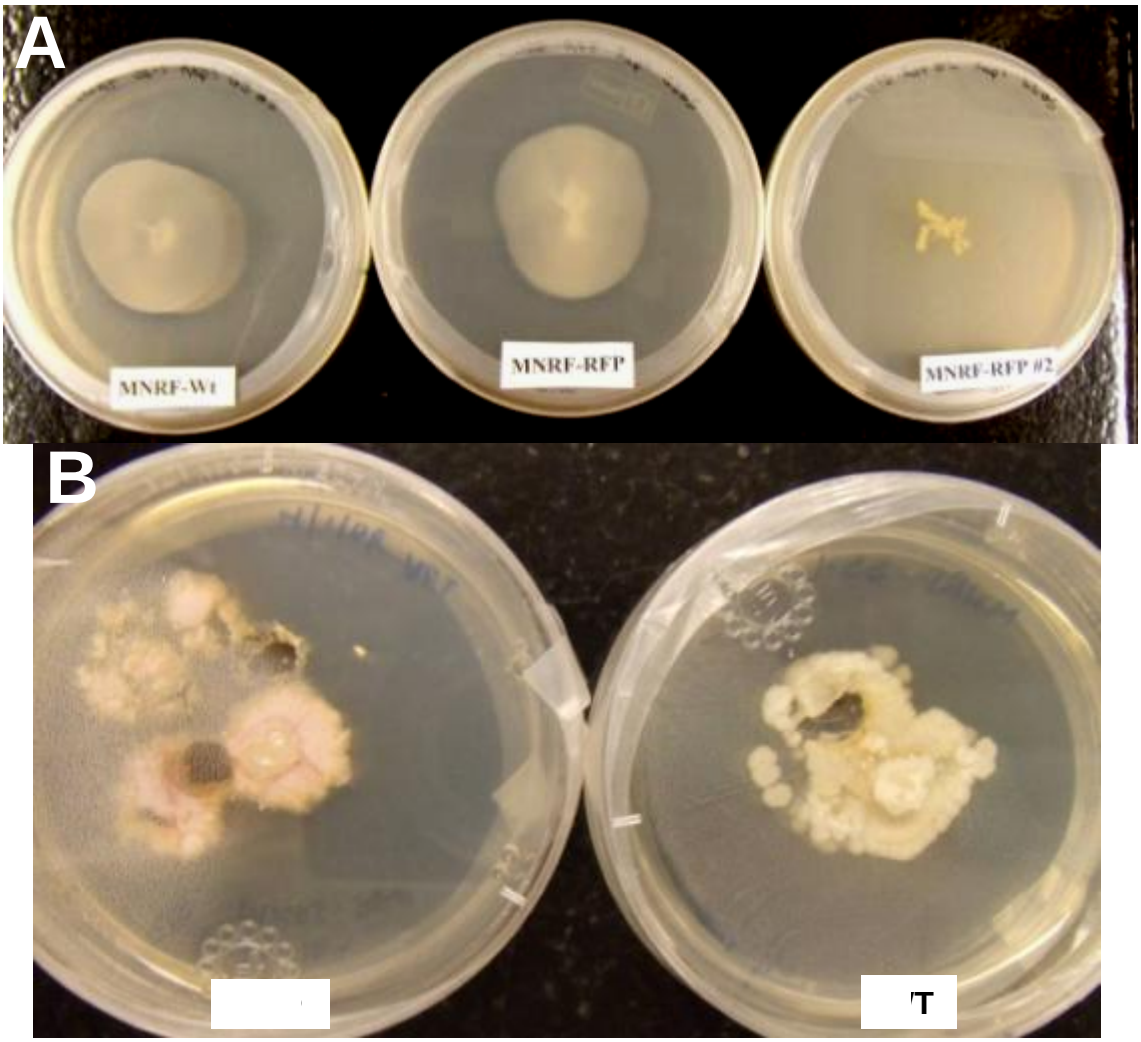

Figure 2.1. **A**) Comparison of growth and phenotype of the wild-type (WT) of *Phialophora gregata* and two isolates transformed to express red fluorescent protein (RFP) on green bean agar. **B**) The red phenotype of *P. gregata* observed after transformation with RFP (left) compared to the off-white WT (right). Cultures were grown on ½x PDA.

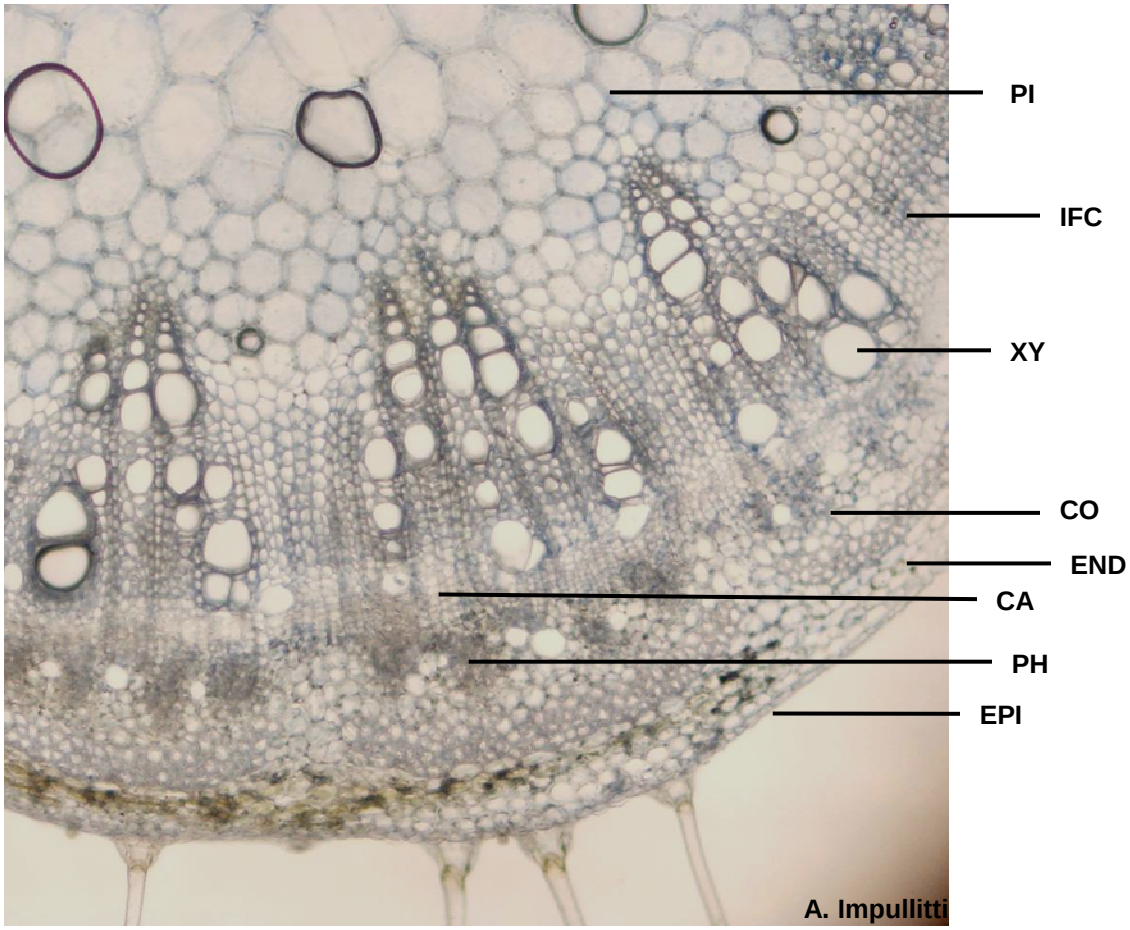

Figure 2.2. Anatomy of a healthy soybean stem. Abbreviations: PI – Pith, IFC – Interfascicular cambium, XY – Xylem, CO – Cortex, END – Endodermis, CA – Vascular cambium, PH – Phloem, EPI – Epidermis (10).

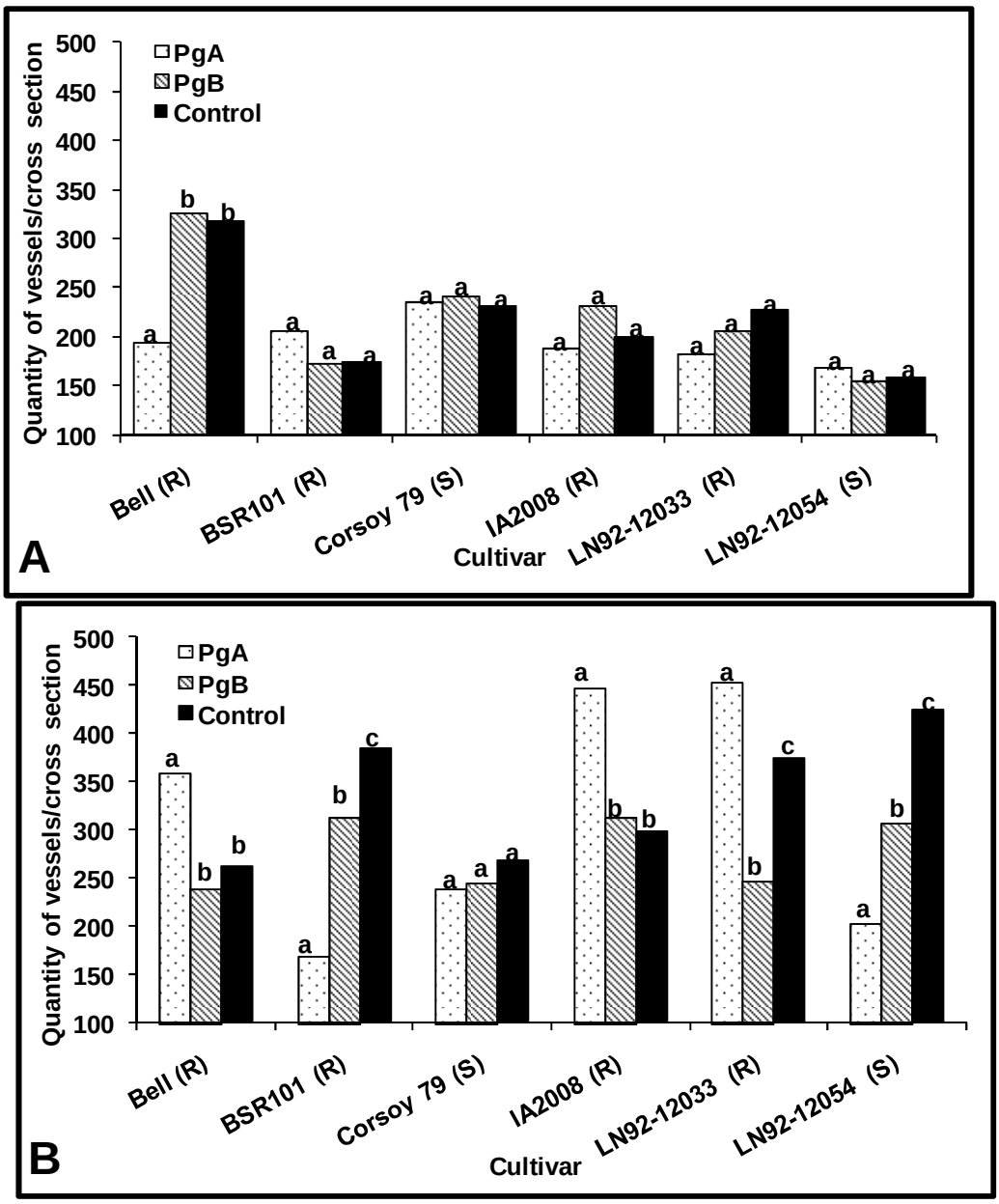

Figure 2.3. Average quantity of vessels per cross section two (**A**) and six (**B**) weeks after inoculation with either Type A (PgA) or B (PgB) of *Phialophora gregata* for six soybean cultivars. Letters in parentheses next to cultivars indicate if the cultivar is resistant (R) or susceptible (S). Data were analyzed by cultivar only. Treatments were compared using an ANOVA followed by a Tukey test, and values with different letters are significant at the $P = 0.05$ level.

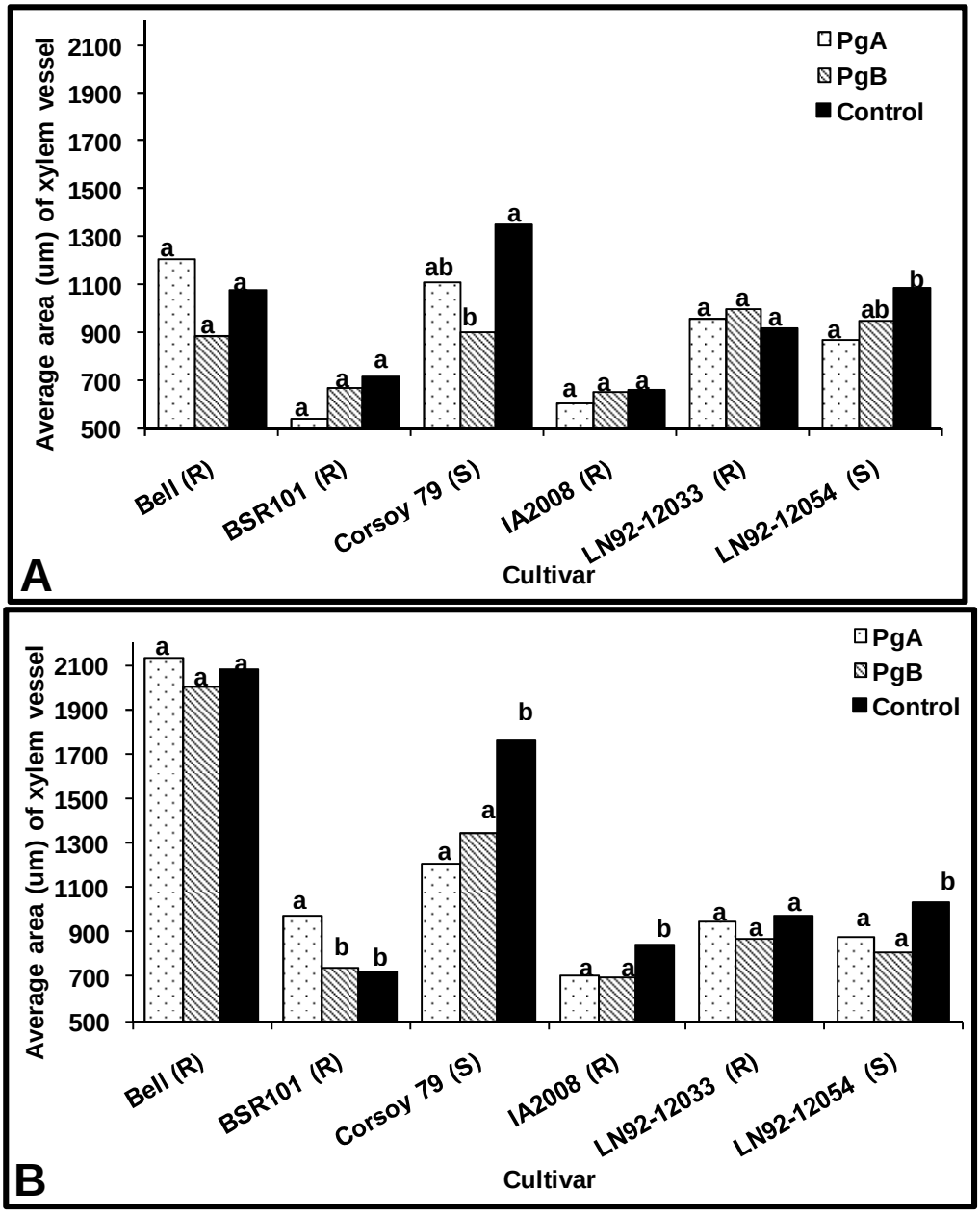

Figure 2.4. Average area (um) of vessels of each cross section two (**A**) and six (**B**) weeks after inoculation with either Type A (PgA) or B (PgB) of *Phialophora gregata* for six soybean cultivars. Letters in parentheses next to cultivars indicate if the cultivar is resistant (R) or susceptible (S). Data were analyzed by cultivar only. Treatments were compared using an ANOVA followed by a Tukey test, and values with different letters are significant at the $P = 0.05$ level.

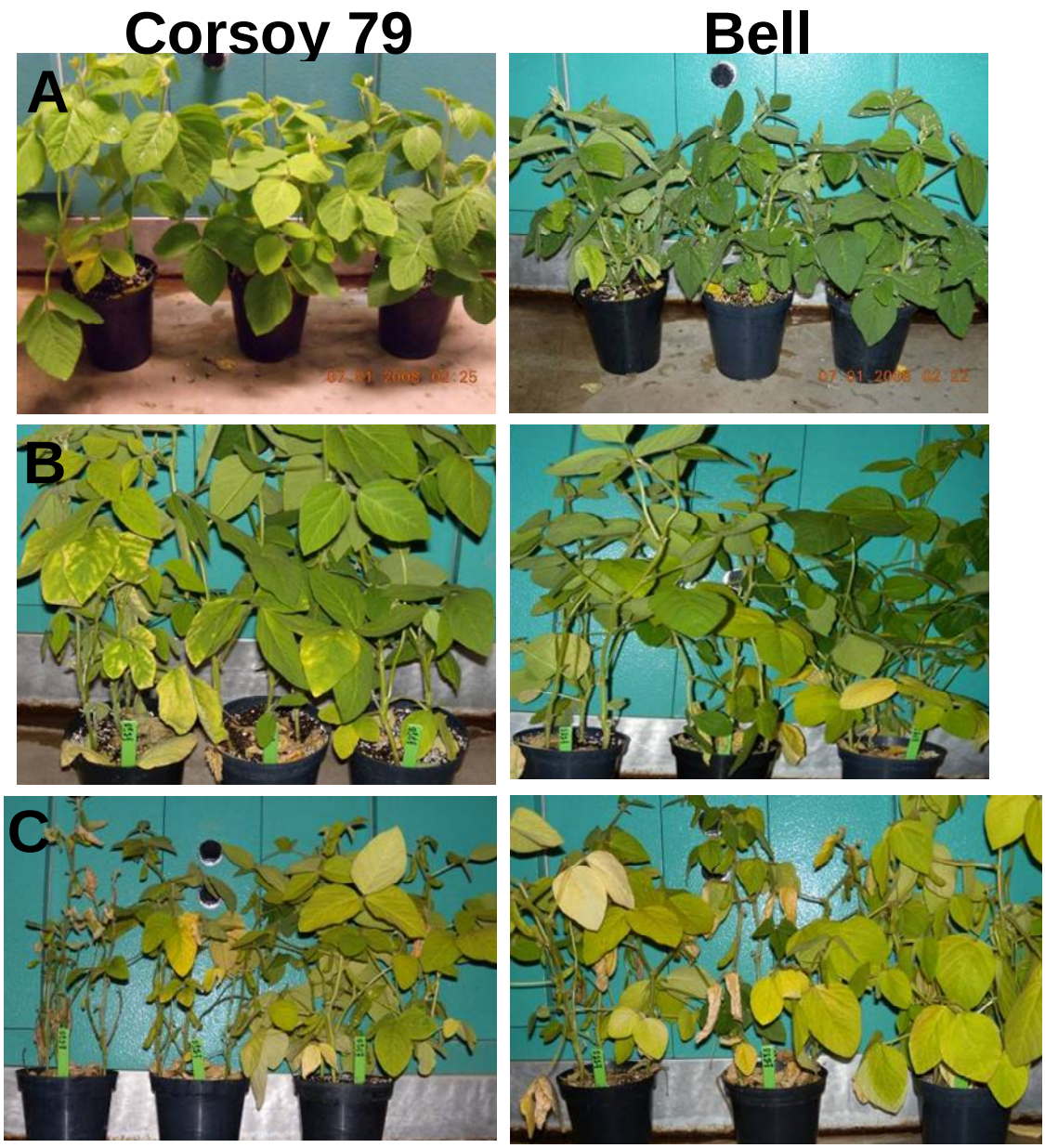

Figure 2.5. The susceptible cultivar, Corsoy 79, and the resistant cultivar, Bell infected with types A and B of *Phialophora gregata* and a control. Photographs shown are from plants 2 (A), 6 (B), and 8 (C) weeks post-inoculation. In each photo, left pot is inoculated with type A, middle pot type B, and right pot is non-inoculated.

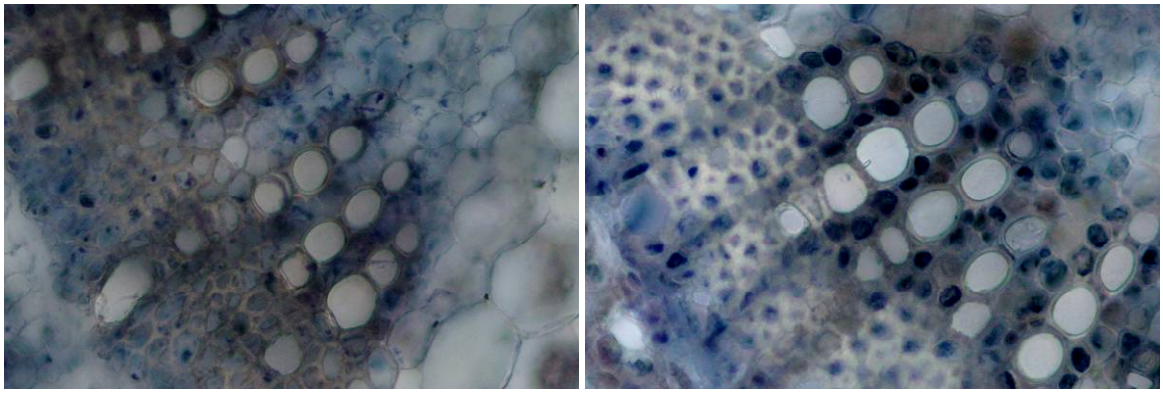

Figure 2.6. A control (left) stem cross section from Corsoy 79 plants (susceptible to BSR) and a plant one week after inoculation with type B of *P. gregata* (right). No macroscopic or microscopic symptoms were observed one week post inoculation, nor were fungal structures in the vascular system of the adjacent tissues or in the other cultivars and treatments at this sampling time (images not shown) (200x magnification).

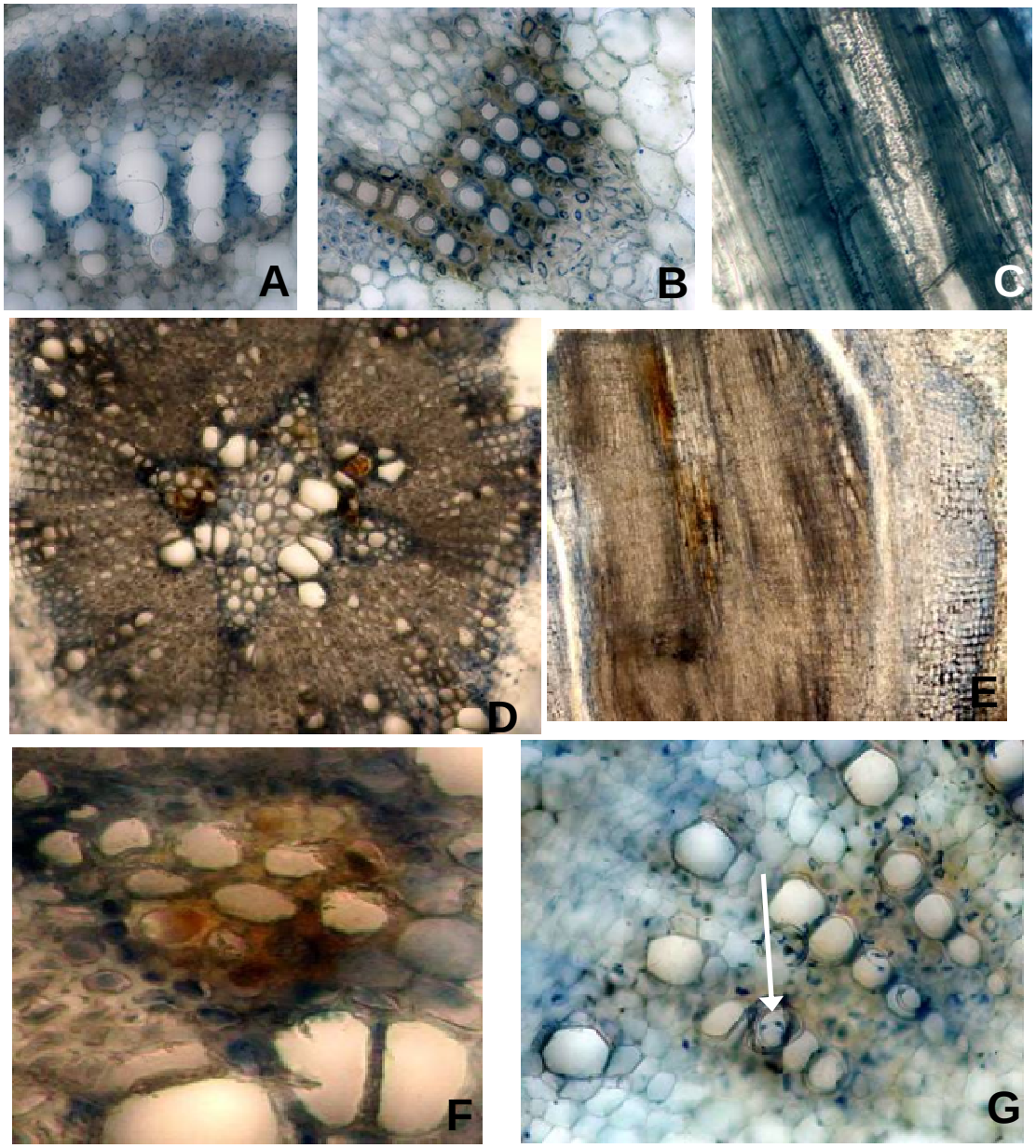

Figure 2.7. Latent infection as observed in stem cross sections from a susceptible cultivar of soybean (Corsoy 79) infected with wild-type isolates of Types A and B of *Phialophora gregata*. Images A-F were captured 2 weeks post inoculation. No colonization was observed in the cross sections of the apex (**A**) or the cross section (**B**) and longitudinal section (**C**) of the middle of the non-inoculated control plant. **D,** Necrosis in the vascular system of the root; **E,** Longitudinal section of a root; **F** A necrotic region from D, cropped and magnification increased. Image **G** was captured 3 weeks post inoculation and hyphae (arrow) are beginning to colonize the xylem vessels. Image A is 40x magnification, B,C, and E – G, 200x magnification, D 100x magnification.

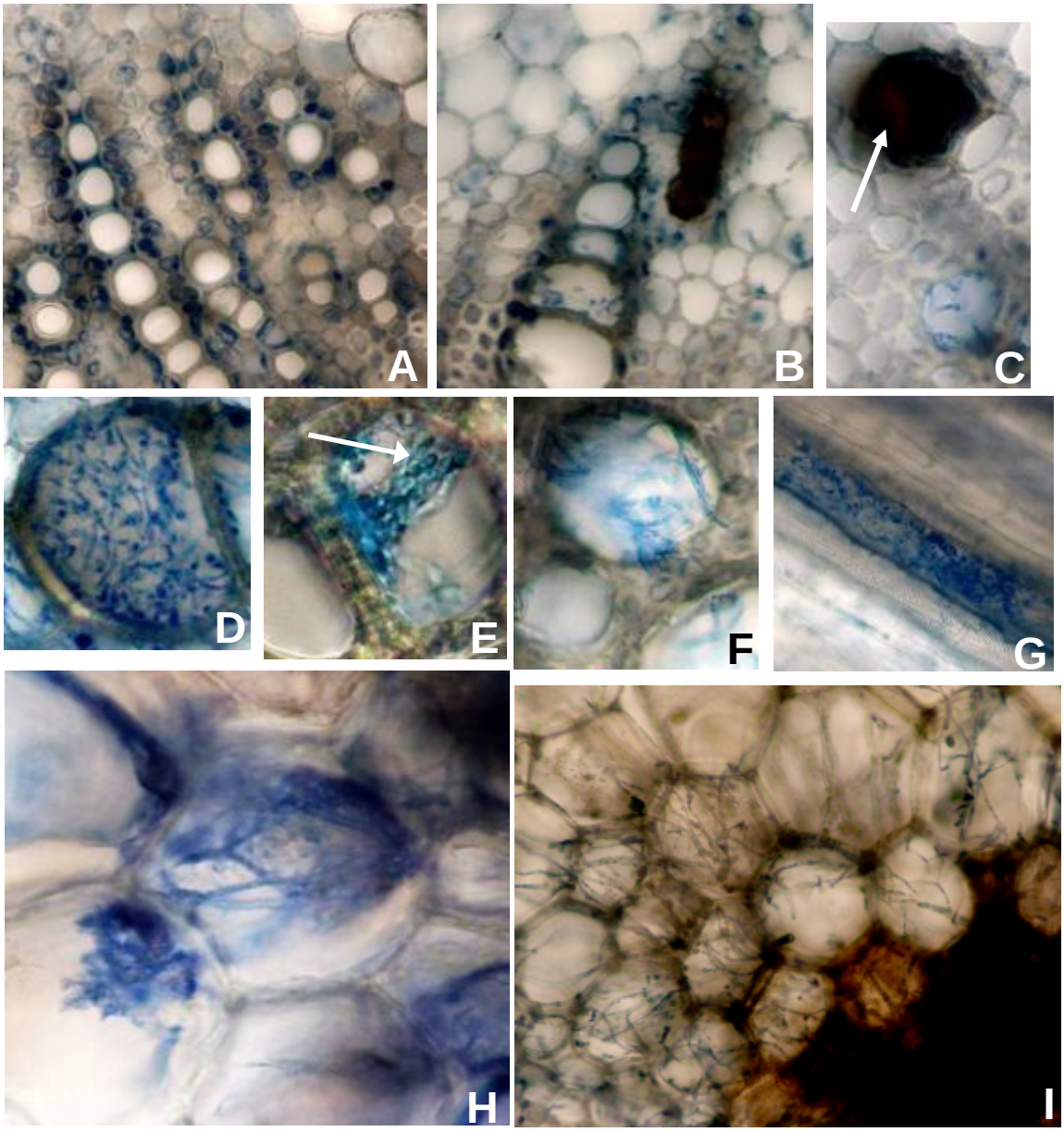

Figure 2.8. Pathogenic infection by wild-type isolates of Type A and B of *Phialophora gregata* as observed in a cross sections of stems from a susceptible soybean cultivar (Corsoy 79). Images were captured 8 weeks post inoculation. **A,** non-inoculated control. **B,** inoculated with Type B. **C,** A necrotic vessel with no fungal structures seen inside **D,** A vessel 100% colonized by *P. gregata*. **E,** A vessel with evidence of sporulation (arrow). **F,** Hyphae of *P. gregata* growing between vessels, possibly via pit pairs. **G,** A longitudinal section of the xylem infected by *P. gregata*. **H and I,** *P. gregata* beginning to colonize the parenchyma cells that compose the pith of the stem. Images A – G and I are 200x magnification, H 400x magnification.

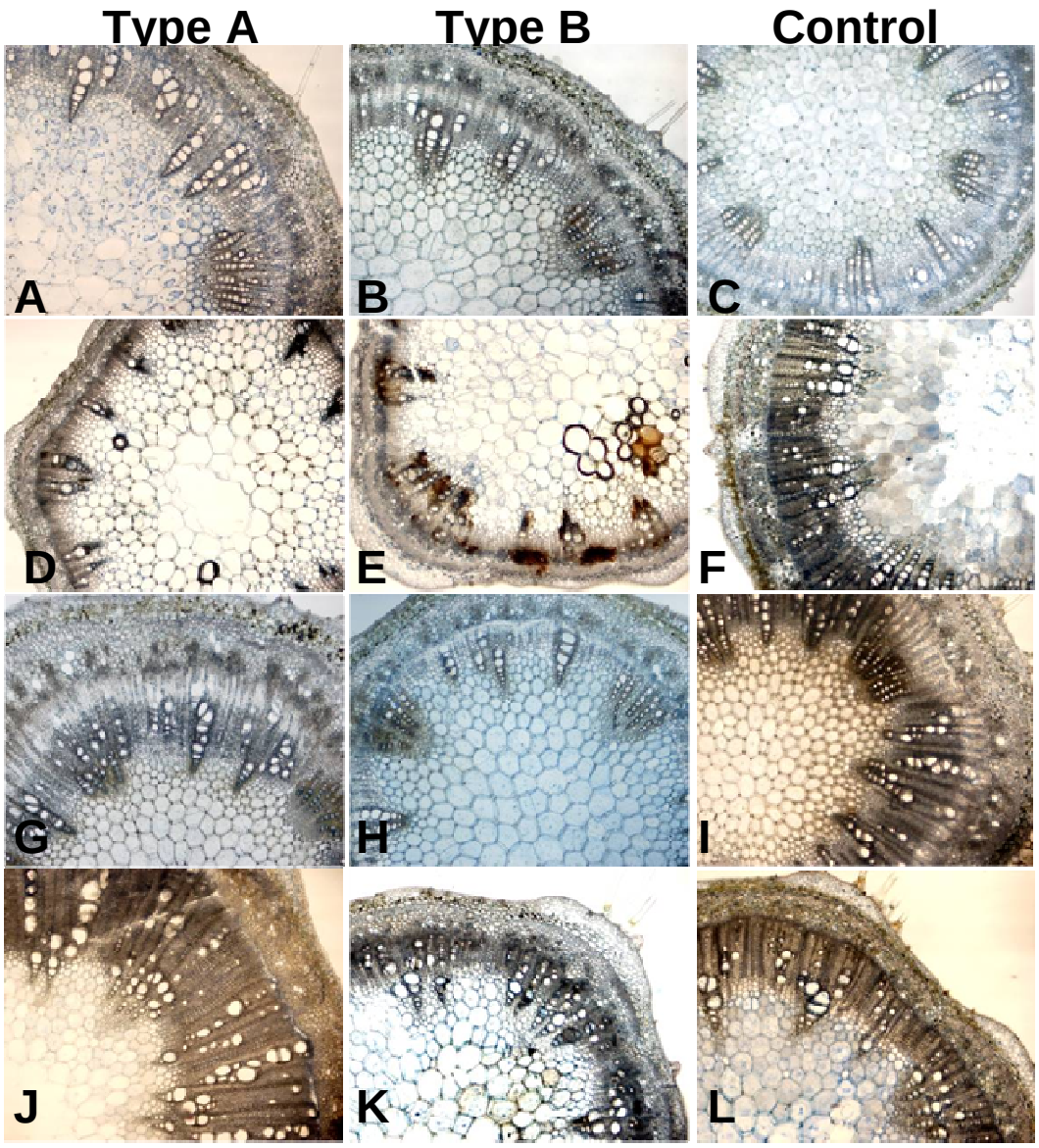

Figure 2.9. The stem anatomy of the resistant cultivar, Bell, and the susceptible cultivar, Corsoy 79, during latent and pathogenic infection by Types A and B of *Phialophora gregata*. During latent infection no differences were seen in anatomical organization in Corsoy 79 inoculated with Type A (**A**), B (**B**) or the control (**C**), but differences in the vascular cambium and xylem structure was seen during pathogenic infection (**D-F**). During latent infection of Bell, the vascular organization was similar in plants inoculated with Type A (**G**) and the control (**I**), but plants infected with Type B (**H**) had less secondary growth and a less pronounced layer of vascular cambium. Differences in anatomical organization were observed during pathogenic infection Bell inoculated with Type B (**K**) had less secondary growth and less of a cambium layer compared to the Bell inoculated with Type A (**J**) and the controls (**F, L**). All images were taken at 40x magnification.

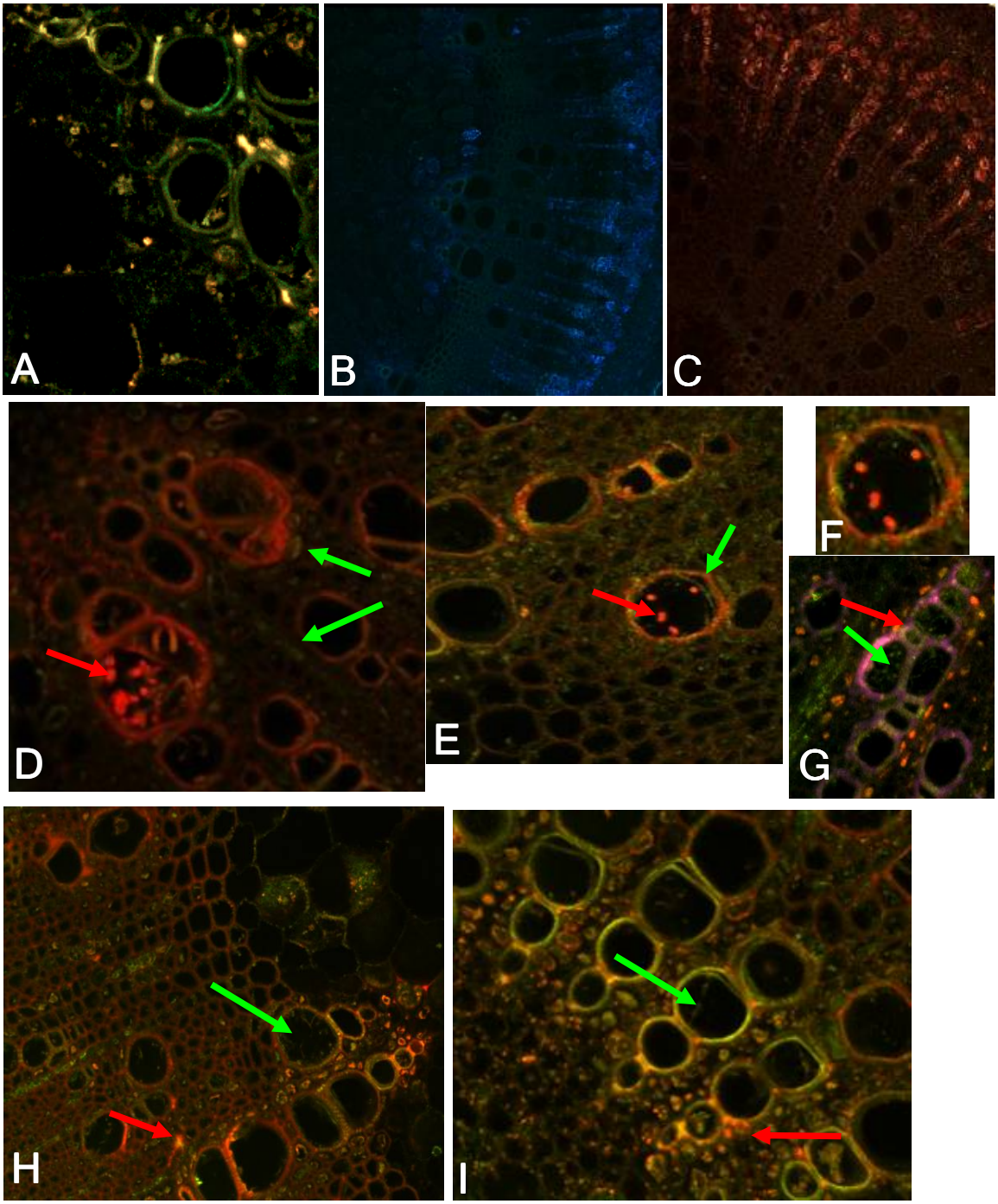

Figure 2.10. Latent and pathogenic infection of stems of Bell and Corsoy 79 either individually or co-inoculated with PgA-GFP or PgB-RFP. (**A**) PgA-GFP infecting a vessel of the resistant cultivar Bell during latent infection. (**B** and **C**) No evidence of fungal infection was observed in the susceptible cultivar Corsoy 79 infected with either PgA-GFP (**B**) or PgB-RFP (**C**) during latent infection. (**D**) Bell co-inoculated with PgA-GFP (green arrow) and PgB-RFP

(red arrow). (**E** and **F**) A xylem vessel of Bell colonized by both PgA-GFP and PgB-RFP. (**F**) is zoomed in for clarity. (**G – I**) Corsoy 79 inoculated with both Types. **A, D – I** 200x magnification; **B, C** 100x magnification.

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Chapter 3: Assessment of fungal endophyte diversity in soybean stems and the potential modification of the mycobiota after the application of glyphosate

The diversity and function of the endophytic community within soybean grown in the U.S. is unknown. The goal of this project was to determine the identity and diversity of endophytic fungi in soybean stems. A secondary goal was to determine if the application of glyphosate modifies the fungal diversity within soybeans. Field-grown, asymptomatic plants of three soybean cultivars either sprayed with glyphosate or untreated were collected as pods began to form on plants. Stems were surface disinfested and either a culture-dependent or -independent approach was used to assess the endophytes. For the culture-dependent approach, stem segments (n = 4000) every 2.5 to 5 cm were dissected into an outer stem composed of the epidermal and vascular tissues and an inner stem composed of the pith tissues. Culturable fungi were grouped based on colony morphology, and then the rDNA ITS region was amplified and sequenced to identify the cultures. For the culture-independent approach, DNA was extracted from the surface disinfested stems, the ITS-region amplified using fungal-specific primers, and then cloned and sequenced for identification. More isolates were obtained from the outer stem segments than the inner stem, and from the base of the plant compared to the apex. The most frequently isolated fungal genera were *Cladosporium* (32%), *Phomopsis/Diaporthe* (15%), *Alternaria* (14%), *Fusarium* (11%), *Phoma* (8%), *Epicoccum* (4%), *Verticillium* (4%), and *Plectosphaerella* (4%). The culture-dependent methods resulted in more endophytic diversity (*H'* = 2.35) compared to the culture-independent methods (*H'* = 0.76). The most prevalent genus identified using the culture-independent method was *Cladosporium* (83%). The genotype of soybean influenced the diversity of endophytes more than glyphosate treatment based on both the culture-dependent and independent methods, and therefore, the application of glyphosate did not affect the diversity of endophytes. Also, this research suggests soybeans harbor an endophytic fungal population much less diverse than plants in the tropics and polycultures.

INTRODUCTION

Endophytes are fungi or bacteria that are ubiquitous in plants and infect asymptomatically (6,31). Although the function and diversity of clavicipitaceous fungal endophytes that infect grasses are well studied, little is known about the function and diversity of non-clavicipitaceous (NC) endophytes in plants, particularly in economically important plants (8,35). NC-endophytes are reported to reduce environmental stress and act as potential biological control agents of plant diseases, and therefore they may be the basis for an emerging approach to improve crop production (4,15,24,26,38).

Soybean (*Glycine max* L. (Merr.)) is adversely affected by many pathogens and environmental stresses, and management of the diseases they cause are often costly, potentially damaging to the environment, and often ineffective. Some soybean pathogen interactions have a latent period that is both similar and distinct from endophytic infection. Latent pathogens and endophytes infect soybean asymptomatically for a period of time without the appearance of disease symptoms, however, unlike endophytes, latent pathogens eventually cause symptoms of disease when conditions are favorable. The identity of latent pathogens of soybean are known, but the diversity and function of the endophytic community within soybean grown in the U.S. is unknown (37). In South America and Asia, bacterial and fungal endophytes in soybean have been identified and characterized (18,20,21,33). Endophytic bacteria isolated from soybean improved plant growth by producing indoleacetic acid, the precursor to auxin (20).

The microbial community in the soil and within plants is potentially modified by the application of herbicides, fungicides, tillage and other agronomic management techniques. A routinely applied herbicide to soybean fields is glyphosate. In 2008, 90% of soybeans grown in the U.S. were resistant to glyphosate. The application of glyphosate to soybean fields is beneficial because it reduces weeds, but modification of the pathogenic and beneficial microbial community has also occurred after its application (40). For example,

the application of glyphosate increased colonization of soybean roots by *Fusarium* sp. and *Pythium* sp., two genera that cause damping-off of seedlings (11,19,27). In another example, disease severity of sudden death syndrome (SDS) increased after application of glyphosate, but *in vitro* growth of *Fusarium virguiliforme* was reduced (36). The influence of glyphosate on the beneficial arbuscular mycorrhizae and *Bradyrhizobium* has also been investigated, but this herbicide did not reduce growth or nodulation by *Bradyrhizobium* or colonization by mycorrhizae (22). In addition, the bacterial endophytic community of soybean grown in Brazil after a pre-plant application glyphosate has also been investigated. The results suggest that some species such as *Herbaspirillum* sp. were more prevalent than others after the glyphosate treatment, while *Xanthomonas* sp. and *Stenotrophomonas maltophilia* were exclusively detected in plants without glyphosate (21).

Despite the fact that the U.S. is the number one soybean producing country, there is no knowledge of the endophyte community in soybean stems. The objectives of this research were to: 1) determine the identify and diversity of endophytic fungi in soybean stems; 2) determine if the application of glyphosate modifies the fungal endophyte population in soybean stems; and (3) to compare the use of culture-dependent and -independent methods for endophyte community characterization.

MATERIALS AND METHODS

Field experimental design and plant collection. Two glyphosate resistant soybean cultivars, AG2107 and MN1803, and a cultivar susceptible to glyphosate, Parker, were used in field experiments in 2008. The cultivar MN1803 is a single backcross of Parker x (Parker x Resnick BC2F2) and was supplied by Dr. J. Orf at the University of Minnesota. Each cultivar was planted on 4 June 2008 in double-row plots that were 2 m long with rows spaced 0.7 m apart in a field site that has a history of a corn and soybean rotation at the Southern Research and Outreach Center in Waseca, Minnesota. Four replicate

plots were planted with the cultivars resistant to glyphosate and two replicate plots with the glyphosate-susceptible variety. Each plot was separated from each other by a minimum of 12 m. Glyphosate (Gly +) was applied at a rate of 1.3 L ha^{-1} on 15 July at the V5/6 growth stage in two of the plots that were planted with AG2107 and MN1803, and two plots remained untreated (Gly -). Six randomly selected plants of each cultivar and treatment without any abiotic or biotic symptoms of disease were excised at the soil line at the R2/R3 growth stage, placed on ice for transport, and then kept at 4°C until processed. In the lab, stems were washed in tap water and leaves removed. The stem length was measured and divided into base, middle, and apex sections. The base consisted of the lower 25 cm, the middle was 26 to 41 cm above the base, and apex was 42 to 58 cm above the base.

Culture-dependent identification of endophytes. Each stem section was washed in sterile water and surface disinfected by immersion in 70% ethanol for 5 s and 10% chlorine bleach (0.525% sodium hypochlorite) for 2 min., then rinsed in sterilized water and dried on a sterile paper towel. Each section was then further divided at 2.2 to 3.8 cm intervals into 0.5 to 0.7 cm segments resulting in approximately 12 segments from each stem for isolation. The segments from the base and mid sections were separated into outer and inner stem segments, and the outer and inner stem segments were each cut longitudinally into four quarters resulting in a total of eight pieces (Figure 1). The inner and outer stem segments of the apex were inseparable. Over 4,000 stem pieces were plated onto 1/2x APDA. Stem pieces were incubated at 25℃ for 5 to 7 days and then transferred to a cold room at 4℃ to restrain fungal growth. Because our goal was to investigate stem endophytes, only the inner stem pieces were analyzed. As mycelium emerged from the stem pieces, a small portion was transferred to 60 mm petri plates containing 1/2x APDA. The plates with no, or minimum growth were checked weekly for 3 months and any fungi present were isolated. Cultures were grouped into 20 morphotaxa based on

colony shape, color, aerial hyphae, growth rate, and texture and were digitally photographed.

Isolates were selected for sequence analysis based on the total number of representatives from each morphotaxa. The number of isolates sequenced was proportional (~1:5) to the abundance of isolates of each morphotaxon. For example, if there were 15 isolates grouped into a morphotaxon, three were selected for sequencing. If there were only five isolates of a morphotaxon, at least two were selected for analysis. Mycelium (10 mg to 70 mg) was removed from each plate with a sterile spatula, and DNA was extracted using the FastDNA® kit (MP Biomedicals, Solon, OH). The intertranscribed spacer region (ITS), including ITS1, 5.8S, and ITS2, of the nuclear rDNA was amplified in a 25 µl PCR reaction containing 1x GoTaq Green Master Mix (Promega, Madison, WI), 0.2 µM of each of the fungal-specific primers ITS1-F and ITS4, 5 µl template DNA, and sterile water using either a MJ Research Thermocycler PTC-100 (Waltham, MA) or an Eppendorf Mastercycler (Westbury, NY) (13,30,42). Negative controls were included for all PCR. PCR products were electrophoresed on a 1.5% agarose gel and viewed after staining with SYBR green to ensure products yielded single bands of appropriate size (ca. 450 to 800 bp). DNA was sequenced as described below.

Culture-independent identification of endophytes. The stem sections that remained after the culture-dependent approach from the base, mid, and apex from each plant and treatment were dried for 48 hrs at 35℃. The inner and outer stem segments became fused and could not be separated after drying. The base, middle, and apex sections were then separately ground using a Wiley® Mini mill (Thomas Scientific, Swedesboro, NJ). Ground tissue was stored at -20℃ until total DNA was extracted using a m odified protocol from the FastDNA® kit (23). The DNA from the base, mid, and apex sections from each plant was then pooled to decrease the number of samples analyzed, and the fungal ITS region was amplified with primers ITS1-F and ITS4 using the same PCR protocol as described above (13). The PCR products from three plants of

each cultivar and treatment were cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. A total of sixty colonies that represented each cultivar and treatment were grown at 37℃ overnight in LB broth amended with 100 µg/ml ampicillin. Plasmid DNA was extracted from clones using a PureLink Quick Plasmid MiniPrep kit (Invitrogen, Carlsbad, CA) and insertion of the rDNA was confirmed by PCR as described above. A total of 300 clones were sequenced.

Sequencing and analysis. DNA that was amplified and isolated from the culture-dependent and -independent approaches was sequenced to identify endophytes. DNA was purified using a PureLink™ PCR purification kit (Invitrogen, Carlsbad, CA). Approximately 80 fungal isolates from the culture-dependent approach were sequenced in one direction using either ITS1-F or ITS4. Five isolates and 10 clones were sequenced in both directions. The complete ITS sequence obtained from the two directional sequencing was used to determine if one directional sequencing resulted in the same taxonomic identification in GenBank. The clones from the culture-independent approach were sequenced using universal M13 primers (28). Half of the clones were sequenced using both directions and half in only one direction. All sequencing was done at the BioMedical Genomics Center (BMGC) at the University of Minnesota – Twin Cities using an ABI Prism 3730xl capillary DNA Analyzer (Applied Biosystems, Foster City, CA).

Sequences ranged from 450 bp to 650 bp and covered the ITS1, 5.8S, and ITS2 regions. Sequences were edited and consensus contigs were determined by the 'assemble automatically' function in Sequencher v4.8 (Gene Codes, Ann Arbor, MI) using 40 nucleotides for the minimum overlap and a sequence similarity value of 99%. Sequence similarities were compared at the 90, 95, 98, and 99% levels. The different comparisons yielded similar consensus groupings, and therefore only the groupings from the 98% level were considered for further analyses. Each consensus sequence generated at 98% similarity was compared with other DNA sequences in the 'nr' database

with Blastn (2). Sequences were considered a match to a genus if the E-value was <0.001, 100% identity, and had an alignment score of > 200.

Assessment of endophyte diversity. The effects of cultivar genotype and glyphosate treatment on endophyte diversity was determined using the Shannon Index to calculate diversity and species evenness. A t-test was used to compare the diversity within cultivars with glyphosate applied and untreated, and to compare the overall influence of glyphosate application on species diversity. Only AG2107 and MN1803 were used in the analysis because glyphosate could not be applied to Parker.

RESULTS

Distribution of endophytes in stems using the culture-dependent approach. Fungal endophytes were isolated from 100% of soybean stems assayed, but fungi were not isolated from every stem segment that was plated. The frequency of isolation was greater and more endophytes were isolated from the outer stem compared to the inner stem, with a total of 1475 and 340 isolates from the outer, and inner stems, respectively (Table 1, Figure 2). AG2107 had the highest isolation frequency of endophytic fungi isolated from 80% and 27%, of the outer and inner stem pieces, respectively, while MN1803 had the lowest frequency of isolation with an average of 60% and 5% (Table 1). The greatest number of isolates were collected from the base of the plants, while 50% fewer isolates were collected from the middle and apex of the plant (Figure 2). From 15% to 50% more isolates were from the basal portion of the inner and outer stem of the AG2107 plants treated with glyphosate compared to the untreated plants (Figure 2). The opposite trend was observed with the cultivar MN1803 where 15% to 50% fewer isolates were detected in the inner and outer lower stem segments of plants treated with glyphosate, respectively, compared to the untreated plants.

Diversity of endophytes in stems. The forward and reverse DNA sequences obtained from five fungal isolates and 10 bacterial colonies

contained the complete ITS rDNA sequence. Comparison of the BLASTn results from the complete and the partial ITS sequence obtained by sequencing in one direction resulted in identification of the same genera (data not shown). Endophytes could not be identified to the species level because multiple species matches were obtained with sequence identity that was greater than 95%.

The culture-dependent methods resulted in the recovery of eleven different fungal genera from the inner stem of soybean that were identified based on >95% ITS sequence match in the Genbank database. The most frequently isolated fungal genera were: *Cladosporium* (32%), *Phomopsis/Diaporthe* (15%), *Alternaria* (14%), *Fusarium* (11%), *Phoma* (8%), *Epicoccum* (4%), *Verticillium* (4%), and *Plectosphaerella* (4%) (Table 2). Two species of *Phoma* and three species of *Fusarium* were also identified. The least common fungi included *Trichoderma* and five unidentified endophytes that did not match any sequences in GenBank (Table 2). Shannon's index of diversity (*H'*) was 2.35 when the 18 different morphotaxa were included.

The culture-independent methods resulted in the identification of seven fungal genera, with the most common genus identified as *Cladosporium* (83%) (Table 2). The remaining six genera only represented 1 to 7% of the sequences. Genera detected that were different from those detected with the culture-dependent methods included *Davidella, Dioscorea*, and *Cryptococcus* (Table 2). The culture-independent methods resulted in less endophytic diversity (*H'* = 0.76) compared to the culture-dependent methods.

Cultivar genotype and endophyte diversity. The cultivar genotype did not have a significant effect on the diversity of endophytes when the results from the culture-dependent and -independent approaches were combined. The diversity was low in both cultivars and the diversity in each cultivar was similar ($P > 0.10$). The two different approaches were also analyzed separately to determine if the cultivar genotype influenced the diversity of endophytes (Figure 3). Diversity was greater using the culture-dependent approach with 13, 9, and

12 genera obtained from AG2107, MN1803, and Parker, respectively, while 6, 4, and 4 ITS genotypes were obtained using the culture-independent approach from the same three cultivars (Figure 3). Parker had the greatest endophytic diversity among the cultivars we evaluated (Figure 3). The most prevalent genera found in all cultivars were *Cladosporium, Phomopsis/Diaporthe*, *Alternaria*, *Fusarium*, and *Phoma*.

Glyphosate and diversity. The application of glyphosate did not have a significant effect on the diversity of endophytes when the culture-dependent and -independent approaches were combined ($P > 0.10$), but differences in diversity were observed when each cultivar was analyzed separately. The diversity of endophytes in AG2107 with and without the application of glyphosate was different using both the culture-dependent and -independent methods ($P < 0.05$), but no differences in diversity were observed in MN1803 using either approach ($P > 0.10$) (Figure 4, data from the independent approach not shown). The glyphosate treatment did not modify the number of isolates of the most prevalent genera detected. Similar numbers of *Cladosporium*, *Phomopsis/Diaporthe, Phoma* sp., and *Fusarium* isolates were detected from plants with and without glyphosate treatments (data not shown).

DISCUSSION

This is the first report describing the diversity and identity of endophytic fungi in soybean stems in the U.S. We used culture-dependent and -independent approaches to determine the diversity of endophytes within stems. We also determined if the use of the herbicide glyphosate modifies the endophytic fungal community within soybean stems. The interaction of endophytic fungi with soybean could be beneficial, negative, or neutral.

Sampling of soybean stems was a critical element of the culture-dependent approach. We isolated endophytes from the base, middle, and apex of the plant, but collected the greatest number of isolates from the base of the stem, and the number declined as samples were collected higher in the plants.

This trend was also previously reported for maize stalks where more bacterial CFUs were isolated from the base than the apex (12). There are multiple reasons why endophytes may colonize the base more than the apex. Most photosynthate travels from the leaves to the base and roots of the plant making the base a nutrient rich habitat ideal for endophyte survival. Also, many of the fungi identified are common in the soil microbial community and could infect plants through the roots and remain close to the root system in the lower stem. Many other fungi are known to be vertically transmitted via the seed, but It was not determined if transmission of these endophytes was due to vertical or horizontal transmission. Fungi that are seed borne or infect plants via the roots may be limited in colonization to the lower stem by the plant or by the growth of the fungus.

Endophytes were more prevalent in the outer stem segments than the inner stem segments. More endophytes were isolated from the outer stem because the plant tissue there is composed of the nutrient rich vascular system and many fungi may be limited to colonization to this area. The inner stem pieces are composed of non-nutritious pith tissues.

The most prevalent genus detected using both the culture-dependent and -independent techniques was *Cladosporium*. *Cladosporium* was also one of the most frequently identified endophytes from the leaves and stems of soybeans grown in Brazil, and from corn grown in Minnesota (30,33). *Cladosporium* is seed borne, but is not reported to be pathogenic on soybean (10). This high frequency of isolation of *Cladosporium* suggests it is the dominant member of the endophytic community and it is a rapidly growing fungus that grew faster than other organisms. Isolates of *C. sphaerospermum* have been investigated for their potential as plant growth promoting fungus. Culture filtrate of an isolate of *C. sphaerospermum* from Korea produced compounds similar to gibberellins and increased soybean biomass and height (14). The species and function of the isolates of *Cladosporium* detected in this

study merit further investigation. Our data and the GenBank database were insufficient to identify the species of this fungus that were present in this study.

The second most frequently isolated genus were members of the *Phomopsis/Diaporthe* complex, which are soybean pathogens that are frequently isolated from stems during reproductive growth stages and can also be seed borne (10,16,37). The *Phomopsis*/*Diaporthe* pathogen complex causes stem canker, pod and stem blight, and *Phomopsis* also causes seed decay. No symptoms of disease caused by these pathogens were observed during this study.

Other fungi that were identified and are known to have an association with soybean, but have not been thoroughly investigated include *Epicoccum*, *Verticillium* and *Phoma* (10). These genera have beneficial and negative associations with other crop plants. Two species of *Epicoccum* are antagonists of *Sclerotinia sclerotiorum* and the effectiveness of *Epicoccum* as a biocontrol agent to reduce white mold has been studied in sunflower and dry beans (17,32). White mold is also an economically important disease of soybean in the north central U.S., and *Epicoccum* could potentially be used as part of a integrated pest management approach to control white mold of soybean (43). *Verticillium* and *Phoma* are common soil fungi and several species are known to infect a wide range of hosts and cause disease.

The diversity of endophytes detected and identified from soybean stems using culture-dependent and -independent methods was less than previously reported from tropical plants and grasses, but similar to the endophytic diversity found in corn in Minnesota (3,25,30). Crops, at least in temperate regions, could have lower endophytic diversity compared to tropical plants and tropical grasses due to monocultures, limited rotations of different crop species, and latitudinal gradients of species diversity. In this study, eleven different fungal genera were identified and five additional unknown “fungal endophytes”. Either the ITS sequences of these unknown fungal endophytes have not been deposited in the database or are truly unidentified species. We were unable to

identify sequences to the species level, which was not surprising due to the misidentification and lack of review of sequences deposited in GenBank (41).

We identified a greater diversity in soybean stems when using culture based methods compared to culture-independent methods, which was unforeseen since the recovery and isolation of fungi using culture techniques is limited by culture media, incubation conditions, and other specialized conditions that some cryptic fungi require. Culture-independent methods to assess the diversity and identify fungal colonizers are becoming more routine, and typically result in more information than traditional isolation and culturing of fungi. Our relatively low number of genera identified using the culture-independent approach could be attributed to PCR bias during cloning (1,5,39,44). When we increased the number of clones sequenced, the number of genera identified did not increase. For example, 50 clones from AG2107 with glyphosate were sequenced. Of these, 1 was *Alternaria* and the other 49 were *Cladosporium*. Another possible issue is that our culture-independent approach used both the outer and inner stem segments. Plant samples were washed and surface disinfested prior to DNA extraction and sequencing, but DNA in dead fungi could have remained on the plant surfaces. Fungi on the epidermis may also have survived the cleaning procedure; however, no common surface contaminants such as *Penicillium* were identified in the culture-independent.

In addition to methodological reasons for low endophyte diversity, there may be other sampling reasons. We targeted stems because others have investigated fungi that infect leaves, pods, and seeds of soybean, and there are more significant stem diseases of soybeans in the north central U.S. than foliar diseases, and therefore we thought there would be a greater potential for discovery of potential biological control agents. We also only studied the isolates from the inner stem. The use of the culture-dependent approach to investigate the outer stem may result in a more diverse endophytic community. Diversity was also low in soybean stems and leaves assayed in Brazil. Only 8 different fungal genera were identified using a culture-dependent approach (33).

Most of the genera identified in the Brazilian study were different than what we isolated in the north central U.S. Genera identified in Brazil, but not the U.S. included: *Colletotrichum*, *Acremonium*, *Aspergillus*, and *Paecilomyces*. Other studies that have investigated fungi that infect soybean have isolated 30 different fungal genera from the leaves, pods, and seeds (29). Many other studies that have investigated diversity in other plants have focused on leaves (4,5,9,25).

The soybean genotype appeared to have a greater influence on the diversity of endophytes isolated than the glyphosate treatment, based on both the culture-dependent and -independent methods. Endophyte diversity in AG2107 differed depending on glyphosate treatment, but MN1803 did not. Most other studies that have investigated the microbial population in soil or roots after the application of glyphosate. No difference in the soil microbial community was found after the recommended rate was applied in a forest or agronomic systems (7,34). However, the species richness of *Fusarium* sp. and bacterial endophytes was altered in soybean following a pre-planting application of glyphosate (19,21).

There is limited knowledge of the endophytic community in soybean, but the composition and function of this cryptic community merits future investigations since many of these fungi could be beneficial or potentially latent pathogens. Future research could identify fungi to the species level, develop and use specific primers to detect their presence in soybean, and determine their function. Other avenues could be to compare the diversity of endophytes in different crop rotations, after the application of fungicides, and in organic production systems.

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Table 3.1. Frequency of endophyte infection and the number of morphotaxa detected from three different soybean cultivars that either had the herbicide glyphosate applied or remained untreated.

	Frequency (%)		Morphotaxa	
Cultivar	**Outer[a]**	**Inner[a]**	**Outer**	**Inner**
AG2107				
+ Gly[b]	82	26	na	13
- Gly	81	29	na	5
MN 1803				
+ Gly	56	3	na	6
- Gly	63	7	na	7
Parker[c]				
+ Gly	na	na	na	na
- Gly	76	11	na	12

[a] Isolates collected from inner and outer stem pieces.
[b] + Gly, application of glyphosate; - Gly, glyphosate not applied.
[c] Parker is susceptible to glyphosate.

Table 3.2. Frequency of different fungi identified using DNA sequencing in three different soybean cultivars using either a culture-dependent approach or a culture-independent approach.

	Frequency (%)	
Genera[a]	**Culture-dependent**	**Culture-independent**
Alternaria	14	4
Cladosporium	32	83
Cryptococcus	0	1
Davidella	0	17
Diaporthe	6	0
Epicoccum	4	0
Fungal Endophyte 1	1	0
Fungal Endophyte 2	1	0
Fungal Endophyte 3	1	0
Fungal Endophyte 4	3	0
Fungal Endophyte 5	1	0
Fusarium sp. 1	6	0
Fusarium sp. 2	4	0
Fusarium sp. 3	1	0
Phialophora	2	0
Phoma sp. 1	4	0
Phoma sp. 2	4	0
Phomopsis	9	0
Plectosphaerella	4	3
Verticillium	4	0
Trichoderma	1	1
ITS Genotypes	**21**	**6**

[a] Genera identified from BLAST based on 98% sequence similarity.

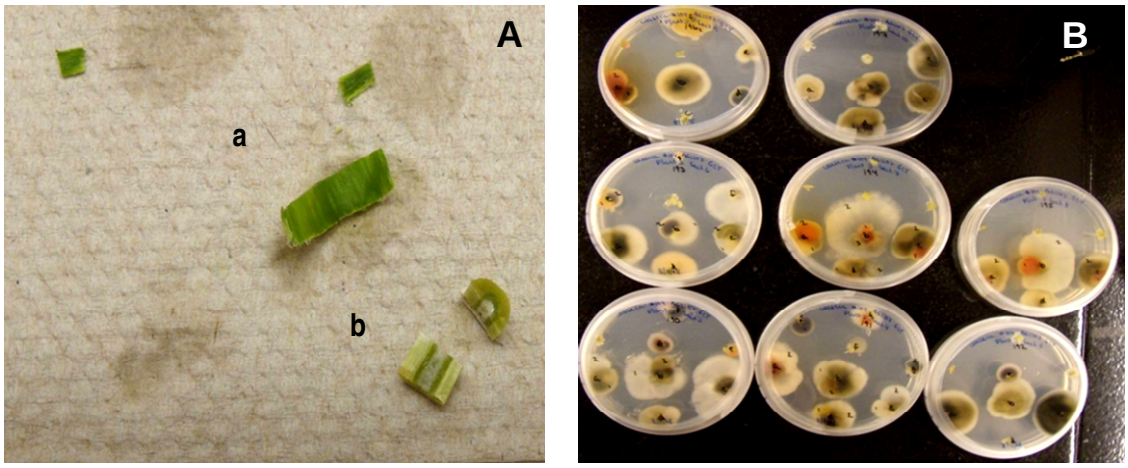

Figure 3.1. A) The outer soybean stem segment (a) and inner stem segment (b) that were used for isolation of endophytes. **B)** Fungi growing out of stems 7 days after placement of tissue on agar-based medium.

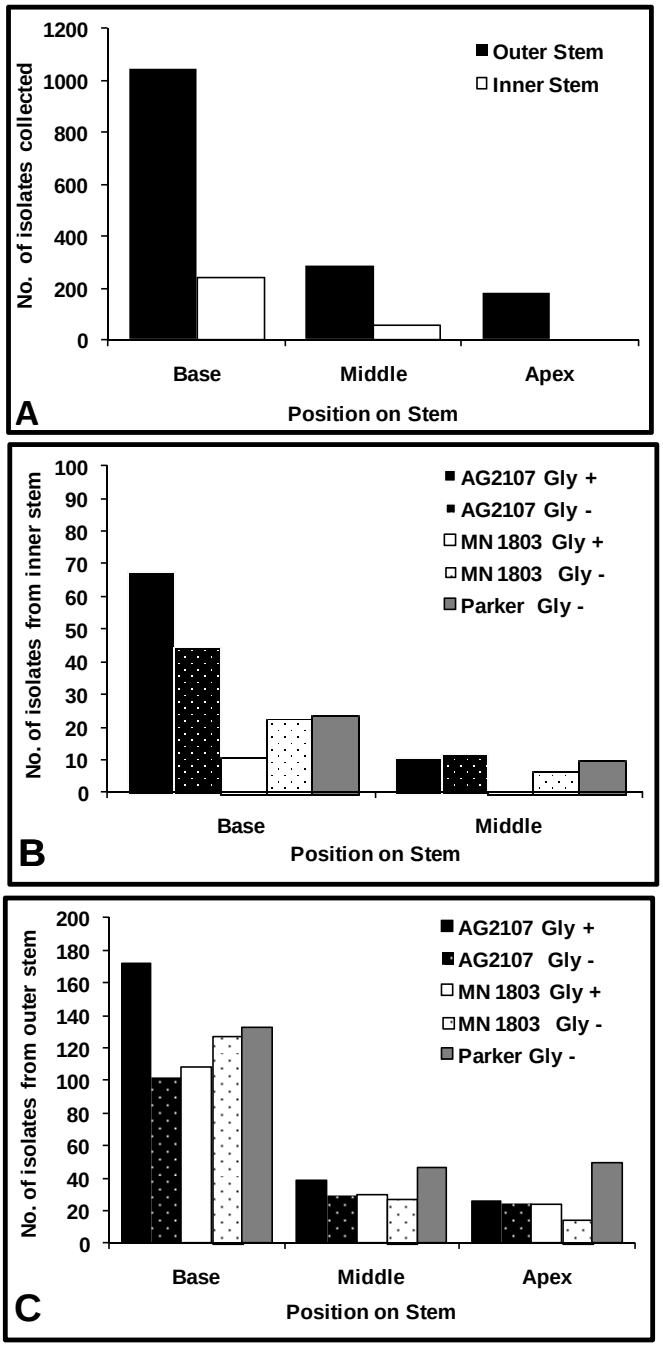

Figure 3.2. The number of isolates from the base, middle, or apex of stems of three different soybean cultivars collected from Waseca, MN. **A**) The total number of isolates collected; (**B**) the number of isolates collected from the inner pith and vascular tissues of the stem; (**C**) and number of isolates collected from the epidermis and cortical tissues of the stem sections. Plants in **B** and **C** were either treated with glyphosate (Gly +) or untreated (Gly -) at the V6 growth stage, and all plants were sampled at the R3 growth stage.

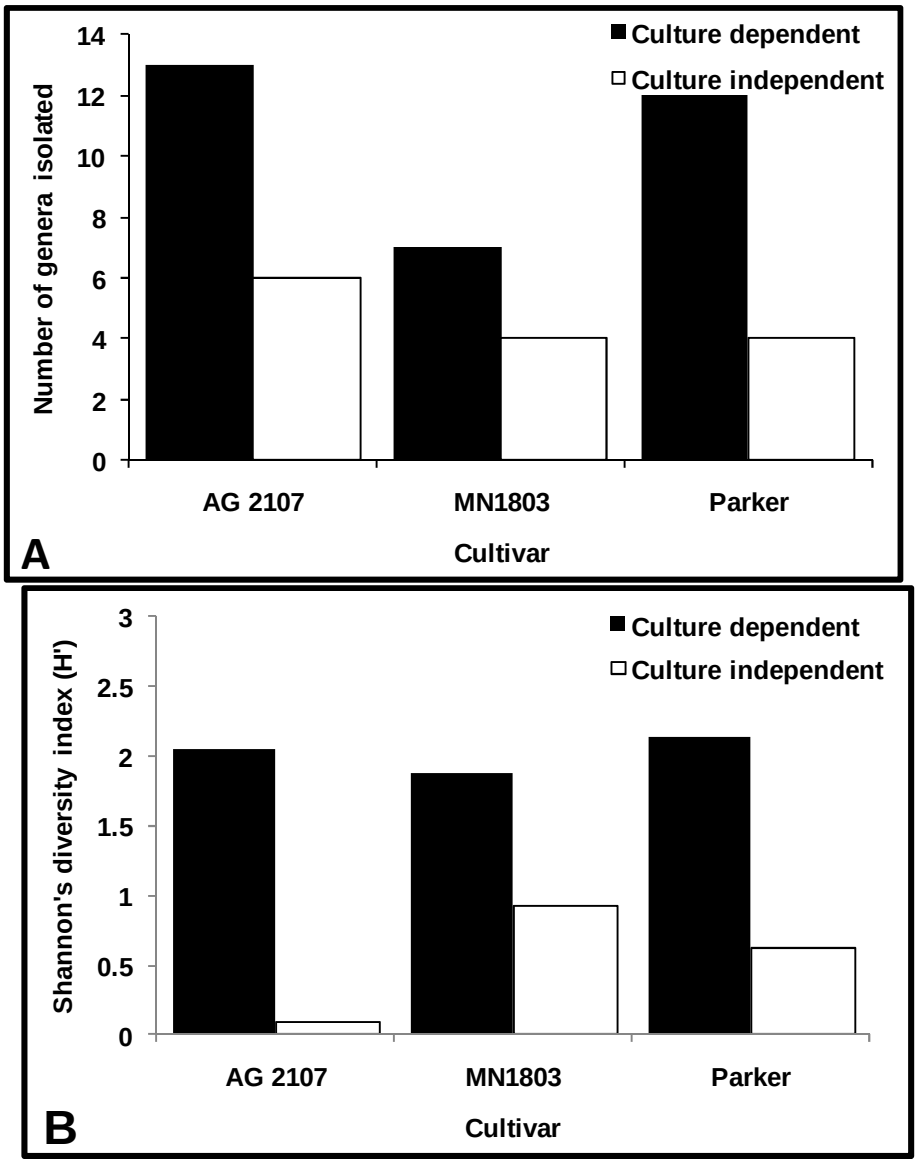

Figure 3.3. A) The number of fungal genera isolated and **B)** the diversity of genera, as measured with Shannon's diversity index, from three different soybean cultivars using culture-dependent and -independent approaches to detect and identify fungal endophytes. For the culture-dependent approach, fungi were isolated from disinfested soybean stems at the R3 growth stage, and for the culture-independent approach, DNA was extracted from soybean stems and the fungal ITS region was amplified and sequenced.

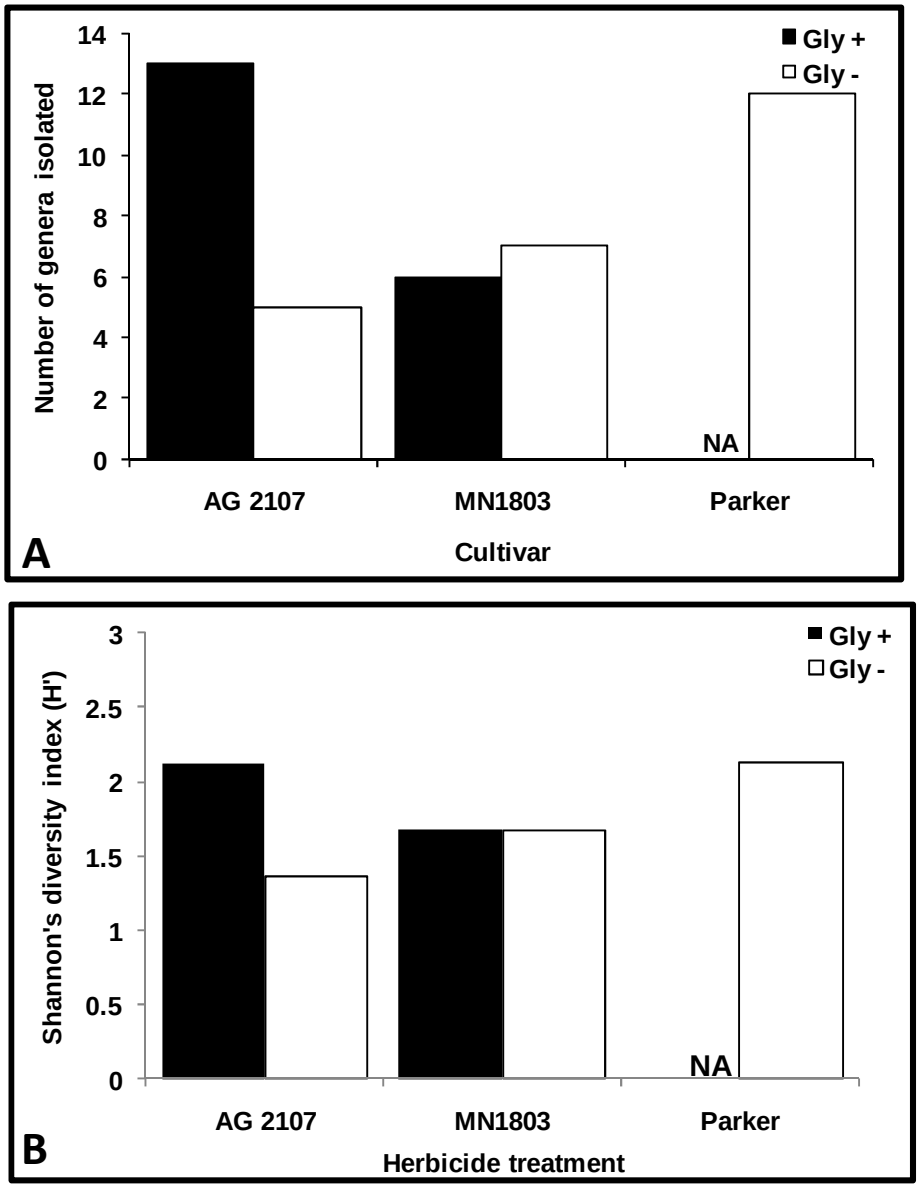

Figure 3.4. A) The number of fungal genera isolated and **B)** the diversity of genera, as measured with Shannon's diversity index, from three different soybean cultivars either treated with glyphosate (Gly +) or untreated (Gly -) at the V6 growth stage, and all plants were sampled at the R3 growth stage. Only data from the culture-dependent approach is shown as results were similar using the culture-independent approach.

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