SEED TO SEEDLING TRANSMISSION OF *COLLETOTRICHUM LINDEMUTHIANUM* IN DRY EDIBLE PINTO BEAN

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MASTER OF SCIENCE

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ABSTRACT

Dry bean anthracnose, caused by *Colletotrichum lindemuthianum*, is a damaging disease that can cause significant reductions in seed quality and yield. The purpose of this study was to determine the races of *C. lindemuthianum* isolates collected in North Dakota and to develop a real-time qPCR assay to quantify the pathogen in seed and stem tissue. Race 73 continues to be the most common in North Dakota across 33 isolates collected in 2012. The qPCR assay using primer pair ClF1527/ClR1609 was proven useful for quantifying *C. lindemuthianum*, and was more specific than a previously developed method. Results from greenhouse and field studies indicated that as seed infection increased, emergence decreased and foliar symptoms and pathogen detection increased. Additionally, field studies demonstrated daughter seed infection increased and yield decreased as seed infection severity increased. Using primer pair ClF1527/ClR1609, the pathogen was detected ten days after planting in greenhouse and field studies.

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LITERATURE REVIEW

The host – dry edible bean

Dry edible bean, or common bean (*Phaseolus vulgaris* L.), belongs to the family Fabaceae, formally known as Leguminosae. This is further divided into subfamily Papilonoideae, tribe Phaseoleae, and subtribe Phaseolinae. The genus *Phaseolus* contains more than 30 species (Siddiq and Uebersax, 2013), five of which are largely cultivated, including *P. vulgaris* L. (common bean), *P. coccineus* L. (runner bean), *P. lunatus* L. var. *lunatus* L. (lima bean), *P. polyanthus* Greenman (year-long bean), and *P. acutifolius* A. Gray var. *acutifolius* A. Gray (tepary bean) (Singh, 2001). Of these five species, *P. vulgaris* is the most cultivated worldwide.

Dry edible bean was first domesticated by the Native Americans during pre-Colombian times and is known to be one of the oldest cultivated crops in America (Broughton et al., 2003; Schwartz et al., 2005). The ancestor of the dry bean was a wild, long slender vine found in Mexico and Central America (Gentry, 1969). Through a process of breeding and selection, the wild vine was refined to the small, dense plant grown today. The biggest differences between the dry edible bean and the wild ancestor are pod and seed size. Large seeds were selected for production, and today's pods contain less fiber (Gentry, 1969). Two independently domesticated gene pools arose – the Andean gene pool characterized by larger seeds and two major phaseolin types ('T' and 'C'), and the Mesoamerican gene pool characterized by small- and medium-sized seeds and the 'S' phaseolin type (Logozzo et al, 2007).

The optimal growth conditions for dry edible beans are in elevated regions of the tropics with average temperatures between 15°C and 24°C (Van Schoonhoven and Pastor-Corrales, 1991). However, dry beans are highly climate adaptable due to their wide genetic diversity and

growth habits. Dry beans have four growth habits. These include Type I – determinate (bush), Type II – indeterminate (vining or trailing) with upright stem and branches, Type III – indeterminate with prostrate (little or no climbing ability) stem and branches, and Type IV – indeterminate with strong climbing ability (Kandel et al., 2013; Singh and Schwartz, 2010). Dry bean seeds are categorized into market classes mainly based on physical appearance of the seed. In the United States, navy, great northern, pinto, cranberry, dark red kidney, pink, small red, small white and black beans are the most commonly grown. Within these market classes, there is large variation in growth habit, phenological traits, seed size, shape, color, and canning and cooking qualities. Genetic diversity of these dry edible beans is divided into large-seed Andean gene pool such as the dark or light red kidney and cranberry and small- and medium-seeded Mesoamerican gene pool such as navy, pink, small red, small white, great northern, and pinto (Singh, 2001).

Dry bean entered the southwestern United States around 2300 B.P. (Brown, 2006; Gentry, 1969; Van Schoonhoven and Pastor-Corrales, 1991). The United States ranks sixth in dry bean production following Myanmar, India, Brazil, Mexico, and the United Republic of Tanzania (FAO, 2014); however, this estimate includes the production of other grain legumes including *P. lunatus* and *Vigna* species. If these other grain legumes are excluded, the United States ranks third as one of the leading common dry bean producers after Brazil and Mexico (Beebe, 2012). In the United States, the top producers of dry beans have included North Dakota, Michigan, Nebraska, Minnesota, Idaho, Colorado, California, and Washington, respectively (USDBC, 2015). North Dakota has been the leading producer of dry beans in the United States since 1991 (Jerardo, 2012). From 2010 to 2013, North Dakota's planted acreage was between 25 percent and 37 percent of the total dry bean acres in the United States (USDA-NASS, 2011;

2012; 2013; 2014). The Northarvest Bean Growers Association, which is comprised of North Dakota and Minnesota, is North America's largest supplier of dry beans. In 2013, pinto and navy beans were the most important market classes in North Dakota, which produced 56 and 38 percent of the national total, respectively (USDA-NASS, 2014); whereas kidney bean is the most important market class in Minnesota, which produced 74 percent of the national total (USDA-NASS, 2014).

Colletotrichum lindemuthianum

Taxonomy and biology. *Colletotrichum* is classified as a coelomycete, known for producing conidia in acervuli (Alexopoulos et al., 1996). *Colletotrichum* was first classified as the genus *Vermicularia* (Tode) Fr. (Sutton, 1992) but was reclassified as *Colletotrichum* due to the presence of setae which are characteristic of this genus, although not all species produce setae. *Glomerella* is the only known teleomorph to be associated with *Colletotrichum* species (Sutton, 1992). The genus *Glomerella* is classified in the Kingdom *Fungi*, Phylum *Ascomycota*, Family *Pyrenomycetes*, and Order *Phyllachorales* (Agrios, 2005; Alexopoulos et al., 1996). *Glomerella lindemuthiana* (Shear), the teleomorph of *Colletotrichum lindemuthianum*, is rarely found in nature (Pastor-Corrales and Tu, 1989) and produces ascospores in perithecia.

Colletotrichum lindemuthianum (Sacc. & Magnus) Lams.-Scrib. produces pale, salmon-colored gelatinous fluid with masses of conidia in acervuli on infected bean seed and aerial tissues of the bean plant. The conidia are unicellular, hyaline, cylindrical, and uninucleate and measure approximately 2.5-5.5 x 9.5-22 µm (Mordue, 1967; Schwartz et al., 2005). Acervuli are intra- and sub-epidermal, disrupting the epidermal cell wall of the plant. Acervuli can grow to about 300 µm in diameter (Mordue, 1967). Brown, septate setae may develop at the edge of an acervulus. In culture, *C. lindemuthianum* grows hyaline to gray hyphae which turn dark to

nearly black with compact aerial mycelium when the fungus reaches maturity. *C. lindemuthianum* may develop sclerotia for survival, although this rarely occurs in nature (Sutton, 1992).

Dry bean anthracnose. Dry bean anthracnose, caused by the fungal pathogen *Colletotrichum lindemuthianum*, is a damaging disease of dry bean that can cause reductions in seed quality due to blemishes, seriously affecting the market value of the seed, and large reductions in yield (Markell et al., 2012; Mohammed and Sangchote, 2007). *C. lindemuthianum*, pathogenic to many legume species including dry edible bean, scarlet runner bean, black gram, mung bean, cowpea, and fava bean, was first described in literature in 1889 (Mordue, 1971). Anthracnose is one of the most important diseases of dry bean and is dispersed worldwide, but causes greater yield loss in temperate and subtropical zones (Balardin et al., 1997). Yield losses can reach 100% when contaminated seed is used, large amounts of inoculum are present, and favorable weather conditions occur during the crop cycle (Holliday, 1980).

Dry bean anthracnose first was detected in North Dakota in 1982 in seed submitted to the NDSU Plant Disease Diagnostic Laboratory (Venette and Donald, 1983). In 2011, 20 percent of dry bean fields in Wells, Eddy, and Ramsey counties had some level of anthracnose (Markell et al., 2012). While widespread losses have not been observed in North Dakota, the lack of host resistance to new races and favorable environmental conditions could lead to substantial economic losses due to this disease. However, anthracnose can cause difficulties in the production of certified bean seed. Since this disease is seed-borne, precautionary steps must be taken to prevent disease onset such as regular field inspections.

Disease cycle. *C. lindemuthianum* is most commonly introduced into a field through planting infected seed. Inoculum for in-season infections can be produced from seed

transmission and on crop residue, resulting in rain-splashed or wind-dispersed conidia. Early infections caused by infected seed can be devastating and have been proven to be the most important factor in disease development (Tu, 1983). Root infection has not been documented, most likely due to the epigeal germination by the dry bean seed (Mohammed and Sangchote, 2005). Unlike other pulse crops such as peas and fava beans, the cotyledon of dry bean grows upward bringing infected plant tissue above the soil level. This type of growth favors pathogens which attack above ground plant tissues (Maude, 1996). Rain and wind will disseminate conidia from infected to healthy hypocotyls which develop sunken lesions, or eyespots, and could lead to the complete destruction of the plant. Wind-dispersed or rain-splashed conidia contact the leaf surface, and the infection spreads. Conidia can spread between fields on contaminated equipment, people, animals, or by stormy weather (Tu, 1988; 1989). Conidial germination occurs within 6 to 9 hours under favorable conditions of temperatures between 13 and 26°C with optimal temperature of 17°C and a relative humidity greater than 92%, or free moisture (Schwartz et al., 2005). Conidia germinate to form germ tubes and appressoria which attach to the host by means of a gelatinous substance. The hypha penetrates the host mainly by mechanical pressure and grows between the cell walls for 2 to 4 days without superficial damage to the host cells. Several days later, the cell walls are dissolved by enzymes leading to water-soaked lesions which turn dark. Fungal mycelium expands from the penetration site, grows to form a lesion, and acervuli are formed which rupture the host cuticle. Conidia formed in acervuli serve as the secondary inoculum and germinate within 6 to 9 hours under favorable conditions. Dry edible beans serve as the only known crop host of the pathogen that is grown in North Dakota (Markell et al., 2012).

Seed to seedling transmission of *C. lindemuthianum* does not always occur, but increases with increasing seed infection, possibly resulting in pre- or post-emergence seedling death. Seed to seedling transmission has been studied under the upper Midwest's alternating dry-wet growing conditions, which can affect the growth of *C. lindemuthianum* in the plant; however, these studies were based only on symptom expression and did not evaluate pathogen presence (Conner et al., 2006; 2009; Mohammed and Sangchote, 2005; 2007; Tu, 1983).

Symptoms. *C. lindemuthianum* can cause infection on all above-ground dry bean tissues. Several days after emergence, infected cotyledons and the first true leaves display small, dark brown to black lesions. Lesions are more common on the abaxial side of the leaf and are long, angular, and brick red to purple in color and follow the leaf vein. Older lesions turn dark brown to black and appear sunken. *C. lindemuthianum* can also infect stems, and lesions on older stems may extend to be 5 to 7 mm long (Allen and Lenné, 1998). Pod lesions appear circular, sunken, and dark colored with a red halo and defined raised edge with many tiny black acervuli containing masses of conidia (Tu, 1988). Young pods shrivel and die if severely infected. Seed infection can occur within infected pods and is characterized by yellowish to brown-black lesions. Early infection can cause the seeds to shrivel, but those infected late in the growing season may not display any symptoms (Agrios, 2005).

Disease management. Prevention is crucial for the management of anthracnose. Ultimately, infected seed is the most common cause of infection in a field since *C. lindemuthianum* can survive in infected seed for up to five years (Markell et al., 2012; Tu, 1983). Seed treatment can help but will not eliminate the chance of seed to seedling transmission. Seed infection alone can result in yield losses ranging from 15 to 32 percent (Conner et al., 2004).

Previous research has documented seed to seedling transmission to be as high as 15 percent in symptomless seed and increases with increasing severity of seed infection (Tu, 1983).

The North Dakota State Seed Department (NDSSD) enforces a zero-tolerance (any level of *C. lindemuthianum* detected results in seed-lot rejection) policy for dry bean seed infected with *C. lindemuthianum* (NDSSD, 2008). The NDSSD has taken measures to ensure dry bean seed produced in North Dakota is free from the pathogen. In 2002, the state began requiring certified seed grown in North Dakota to be tested for anthracnose. The use of certified, clean seed from fields that have been field inspected and tested post-harvest for this pathogen is recommended (NDSSD, 2008).

Crop residue left from the previous two growing seasons may act as a source of *C. lindemuthianum* for current season infection of dry beans; therefore, crop rotation to non-host species can aid in managing anthracnose spread across seasons (Markell et al., 2012; Tu, 1983). Tillage practices have shown mixed results for the management of dry bean anthracnose. Tilling infested crop residue will reduce the risk of disease transmission to dry beans in adjacent fields or to the next year's crop; however, studies have shown the pathogen can survive for one to two years when buried in soil, and when tillage returns the residue to the surface, disease transmission can occur from the previously buried residue (Dillard and Cobb, 1993). A four-year rotation is recommended to allow crop debris and potential inoculum to decompose. Limiting dry beans planted next to fields infected the previous year can aid in delaying or avoiding infection, as rain-splashed or wind-dispersed conidia produced on debris can cause new infections (Tu, 1988; 1989). Because of this, it is important to stay out of wet fields to avoid the potential of spreading conidia from plant to plant.

Fungicides have limited economic benefit, mainly because application timing is critical to disease management (Conner et al., 2004; Tu, 1992; 1996). Foliar fungicide application timing revealed that the most consistent results were obtained when pyraclostrobin was applied twice, once during early bloom and once during late bloom (Conner et al., 2004). A single application of pyraclostrobin was nearly as effective and significantly increased yield; however, the optimal timing for this single application, either early bloom or late bloom, was not clear due to variations in the weather. When anthracnose symptoms are clearly present, a fungicide application after flowering will not prevent losses in seed yield and quality.

C. lindemuthianum races and race identification. The use of dry bean cultivars with resistance to *C. lindemuthianum* has been an effective management practice, but numerous races of *C. lindemuthianum* exist and the pathogen race structure has changed throughout time. The appearance of new races may result in a change in race prevalence across the region and could overcome available resistance that is relied upon for the control of this pathogen. Variability in the species was first observed when races α and β were identified based on their reactions on 139 bean cultivars (Barrus, 1911; 1918). Until 1994, many countries used local hosts and local codes rather than Greek letters to identify races (Melotto et al., 2000). Today, a standard series of 12 dry bean differentials with diverse origin evaluated with a binary scoring system is used to identify races and describe the variability within *C. lindemuthianum* (Melotto et al., 2000). The susceptible cultivars' binary number sum will give the race number of *C. lindemuthianum* (Pastor-Corrales, 1991). This standard procedure has allowed for the comparison of data from different research groups and easier characterization of *C. lindemuthianum* races.

Changes in race structure of the pathogen population have been recently observed in dry bean growing regions worldwide (Campa et al., 2009; 2011; Damasceno e Silva et al., 2007; del

Río et al., 2003; Goswami et al., 2011; Pastor-Corrales et al., 1995). In North Dakota, *C. lindemuthianum* races 7 and 89 were detected in 1994 (Kelly et al., 1994), and race 73 was detected in 2001 (del Río et al., 2002). In surveys performed in 2008 and 2009, two new races 1153 and 1161 were detected for the first time in North Dakota and North America (Goswami et al., 2011). However, race 73 remains the most commonly isolated in North Dakota. Isolates of these new races have the capability to infect dry bean cultivars of different market classes with varying degrees of disease severity. With this potential risk, it is very important for imported and exported seed to be tested for the presence of *C. lindemuthianum* to help prevent field infections and the introduction of new races. Pathogen races are most commonly determined by screening isolates against the previously mentioned set of differentials known to have one or more of the 13 major resistance genes. Although the screening of germplasm with a bulk of races can provide useful information, such as which resistance genes will not work against the pathogen population for dry bean breeders, true race identity using single-spore cultures is necessary for evaluation of pathogen diversity.

Breeding for anthracnose resistance is complicated by the existence of several physiological races of the pathogen; however, breeding can be aided by pyramiding major resistance genes for long-term resistance in *P. vulgaris* (Young and Kelly, 1997). Genetic resistance is controlled by major resistance genes acting individually, complementary, or as member of an allelic series (Melotto et al., 2000; Young and Kelly, 1996; 1997; Young et al., 1998) conferring protection against specific races of the pathogen. The use of single resistance genes is not recommended because the pathogen can build resistance more quickly. Stacking major genes may be the best strategy for breeding resistance in *P. vulgaris* because breeding several resistance genes could provide more stable anthracnose resistance (Melotto et al., 2000).

Currently, the differential cultivar 'G 2333' has three resistance genes – *Co-4*^{2}, *Co-5*, and *Co-7*. Of these three, *Co-4*^{2} has become a gene of interest. Research has shown that this gene has resistance to 97 percent of the *C. lindemuthianum* races present in North and South America (Balardin and Kelly, 1998). Research is being performed to map this resistance gene which encodes a protein kinase (Broughton et al., 2003; Melotto and Kelly, 2001). Further understanding of this resistance gene and others will allow scientists to better understand the evolution of the pathogen and enable studies regarding plant-pathogen interactions.

In planta quantification of *C. lindemuthianum*. Traditionally, anthracnose of dry bean has been diagnosed via pathogen isolation from infected tissues using nutrient agar. Visual detection of morphological structures diagnostic of *C. lindemuthianum* can take 14 or more days to complete (ISTA, 2008). Distinguishing anthracnose from other diseases displaying similar symptoms, such has common bacterial blight or halo blight of dry bean, can be difficult without the presence of fungal growth and identification. These methods are labor intensive and may not be sensitive enough to detect symptomless seed infections. If the pathogen is not visually detected, the tissue or seed sample will be deemed disease-free, possibly falsely.

Real-time qPCR assays provide a rapid, accurate and specific method of plant pathogen detection in water, soil, air, and plant tissue (Okubara et al., 2005). A single fragment of target DNA can be amplified selectively from infected tissues with the use of specific oligonucleotide primers, eliminating the need for pathogen isolation (Henson and French, 1993; McCartney et al., 2003). Real-time qPCR has been used to quantify the extent of seed infection for fungal pathogens in a number of cereal, fruit, and oilseed crops (Bates et al., 2001; Cottyn et al., 2011; Cullen et al., 2002; Debode et al., 2009; Guillemette et al., 2004). Target DNA is quantified by relating the intensity of fluorescence to known amounts of template DNA in a standard curve.

Various regions of DNA have been used to design real-time qPCR primers for pathogen detection including nuclear ribosomal DNA (rDNA), internal transcribed spacer (ITS) regions, and beta-tubulin genes. The nuclear rDNA usually encodes for genes needed for protein synthesis and tends to be conserved within and between species. The ITS regions are the spacer DNA between the rDNA genes, tend to be less conserved, and are found in multiple copies within the nuclear genome. Having multiple copies of these regions reduces selection pressure and allows mutations to occur and accrue particularly in the ITS regions. Sequence variations and multiple copy numbers cause difficulties when trying to develop primers and probes to quantify a species in question (McCartney et al., 2003). Primers designed from the ITS regions can be effective for phylogeny studies and detection of an organism. However, the conserved regions of other nuclear genes such as the beta-tubulin gene allow for the development of better probes and primers for the quantification of a specific species because they are conserved throughout the evolution of an organism (McCartney et al., 2003).

A PCR-based system was developed for the detection of *C. lindemuthianum* in symptomatic and asymptomatic seeds and infected plant leaves using nested PCR (Chen et al., 2007). Five forward primers were designed from the ITS1 region to be used with universal primer ITS4. Of these five forward primers, ClF4 and reverse primer ITS4 produced a 461 base pair amplicon specific to *C. lindemuthianum* (Chen et al., 2007). Further testing using nested PCR allowed the detection of as little as 10 fg of the pathogen DNA mixed with host DNA. Soon after, another PCR-based system was developed (Wang et al., 2008). Two primer pairs (CY1/CY2 and CD1/CD2) were used in a duplex conventional PCR assay. These primer pairs produced products that were 442 and 638 base pairs, respectively, and were unique to *C. lindemuthianum*. The duplex PCR assay was developed to separate *C. orbiculae*, the causal

agent of anthracnose on cucurbits, from *C. lindemuthianum* due to the similarities of the ITS sequences of these two organisms. Later research resulted in the development of a real-time qPCR-based assay for the detection of *C. lindemuthianum* in dry bean tissue (Chen et al., 2013). This real-time qPCR assay utilized SYBR green dye and was able to detect as little as 5 fg of pathogen DNA. A TaqMan assay utilizing the same primers and probe ClP442 were deemed equally as sensitive. The primers ClF432/ClR533 amplify a 141 base pair fragment of ITS region 2 (Chen et al., 2013). Only the reverse primer, ClR533, located between the ITS2 region and the 28s gene, was highly specific for *C. lindemuthianum,* a product was not produced using this primer from other *Colletotrichum* species and bean pathogens. The primer set was utilized to study the relationship between the level of symptoms on bean seed, including asymptomatic, and the amount of pathogen DNA in bean seed. Results indicated that the lesion size on symptomatic seed strongly correlated with the amount of pathogen DNA detected by the real-time qPCR assay. However, only a weak fluorescent signal was detected in asymptomatic bean seed (Chen et al., 2013). No clear indication was provided to determine if the weak fluorescent signal from asymptomatic seed could be clearly distinguished from background or healthy seed.

Real-time qPCR assays provide a more efficient and effective diagnostic tool for evaluation of the host-pathogen interaction and allow quantification to be completed within two to three days instead of the laborious 14 or more days needed to complete a traditional detection assay. Additionally, real-time qPCR has the ability to detect symptomless infections at a much higher frequency than that observed through traditional methods of detection, and misidentification occurs much less frequently (McCartney et al., 2003). If molecular detection can be validated in a seed lot test for *C. lindemuthianum,* these advancements in detection technology may provide North Dakota dry bean seed growers the ability to market seed against

competitors from regions where the pathogen has not been detected and commercial growers the confidence to know that the seed they purchase is disease-free. Molecular detection may also provide insights to pathogen biology and etiology by enabling more rapid and intensive evaluations for host tissue colonization.

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CHAPTER 1. CHARACTERIZATION OF PATHOGEN RACE TYPES OF ISOLATES OF *COLLETOTRICHUM LINDEMUTHIANUM* COLLECTED IN NORTH DAKOTA

Introduction

Dry bean anthracnose, caused by the fungal pathogen *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib., is a damaging disease of dry bean that can cause seed color blemishes, reduced quality, large reductions in yield, and seriously affect the market value of the seed (Markell et al., 2012; Mohammed and Sangchote, 2007). This disease can affect all foliar parts of dry bean plants. Symptoms include long, angular, and brick red to purple lesions that follow the leaf vein. Lesions appear on the abaxial side of the leaf more often than the adaxial side as a result of wind-dispersed or rain-splashed conidia coming in contact with the underside of the leaf first (Schwartz et al., 2005). Older lesions turn dark brown to black and appear sunken. The stems and petioles also can be affected (Allen and Lenné, 1998). Pod lesions appear circular, sunken, and dark colored with a red halo and defined raised edge with many tiny acervuli containing masses of conidia (Tu, 1988).

Anthracnose is one of the most important diseases of dry bean and is dispersed worldwide, but causes greater yield losses in the temperate and subtropical zones (Balardin et al., 1997). Relatively cool and humid conditions favor the development of this disease. Yield losses can reach 100% when contaminated seed is used, a large amount of inoculum is present, and favorable weather conditions occur during the crop cycle (Holliday, 1980). Dry bean anthracnose is primarily controlled by the use of clean seed along with crop rotation, application of fungicides, and the use of resistant cultivars.

Dry bean anthracnose first was detected in North Dakota in 1982 from seed submitted to the NDSU plant disease diagnostic laboratory (Venette and Donald, 1983). In 2011, 20 percent

of dry bean fields in Wells, Eddy, and Ramsey counties had some level of anthracnose (Markell et al., 2012). While widespread losses have not been observed in North Dakota, the lack of host resistance to new races and favorable environmental conditions could lead to substantial economic losses due to this disease.

Many races of *C. lindemuthianum* have been found worldwide. Mexico reported the existence of 38 races, 7 races were found in Nicaragua, 33 races were identified in Colombia, whereas at least 6 have been reported in the United States (Balardin et al., 1997; del Río et al, 2002; Goswami et al., 2011). These reported races can be broken into two major groups: those found over wide geographic areas and those restricted to a single country (Melotto et al., 2000). Those found over a wide geographic area include *C. lindemuthianum* races 7, 65, 73. It is unknown whether this is due to repeated evolution of the pathogen or due to efficient seed-borne dispersal between areas (Pastor-Corrales and Tu, 1989). Additionally, research has suggested *C. lindemuthianum* races tend to be more diverse in Central America than either South or North America. This is consistent with previous research which reported pathogen races were more diverse in Mesoamerica compared to those from the Andean region (Balardin et al., 1997; Pastor-Corrales, 1996; Sicard et al., 1997). This may be due to the origin of the wild bean ancestor in Central America and the coevolution of the pathogen and the host.

In North Dakota, *C. lindemuthianum* races 7 and 89 were detected in 1994 (Kelly et al, 1994), and race 73 was detected in 2001 (del Río et al, 2002). New races detected in a North Dakota dry bean survey conducted from 2003 to 2009 included 1153 and 1161, which were also reported in North America for the first time (Goswami et al., 2011). Among these, race 73 continues to be the most commonly isolated in North Dakota (Goswami et al., 2011). The appearance of new races may result in a change in race prevalence across the region. In

Manitoba, Canada, race 105 has been found and has a virulence pattern similar to race 73 (Dongfang et al., 2008). These races have the capability to infect dry bean cultivars grown in the area with varying degrees of disease severity. Therefore, it is important to assess the races present throughout the state. The objective of this study was to determine the pathogen races of *C. lindemuthianum* isolates collected in North Dakota in 2012 and 2013. This evaluation will determine if the race structure of the *C. lindemuthianum* population in North Dakota has changed since the last evaluations were performed on isolates collected from 2003 to 2005 and 2007 to 2009 (Goswami et al., 2011).

Materials and methods

Sampling. In the summers of 2012 and 2013, a foliar disease survey was conducted, and samples of infected dry bean leaves, stems, pods, and seeds were collected from fields located in the northeastern section of North Dakota (Fig. 1.1). These samples were collected during the middle of August when the crop was in mid-seed fill to harvest maturity (R6 to R8; Table 1.1).

Pathogen isolation. Samples brought back to the laboratory were surface disinfested by soaking the plant tissue for one minute in a 10% sodium hypochlorite solution followed by three rinses in sterile distilled water. The infected tissue was allowed to hydrate on moist filter paper for 24 h at room temperature and tissue surrounding a single lesion was excised and crushed to loosen the conidia. Loose conidia were streaked onto acid potato dextrose agar (AcPDA) (19.5 g/500 ml distilled water plus 30 µl/500 ml of 27% lactic acid) with 10 ml/500 ml of 30% streptomycin using an inoculating loop. The fungus was allowed to grow for 2 to 3 days in the dark, and hyphal-tip isolation methods were utilized to establish a pure culture on PDA. Samples with symptoms of anthracnose were obtained from nine fields in Wells County in 2012 (Fig. 1.1). In addition, eight isolates recovered using single-spore techniques were provided by

Dr. Michael Wunsch at the NDSU Extension Research lab based in Carrington, North Dakota (CREC) in 2012 (Fig. 1.1).

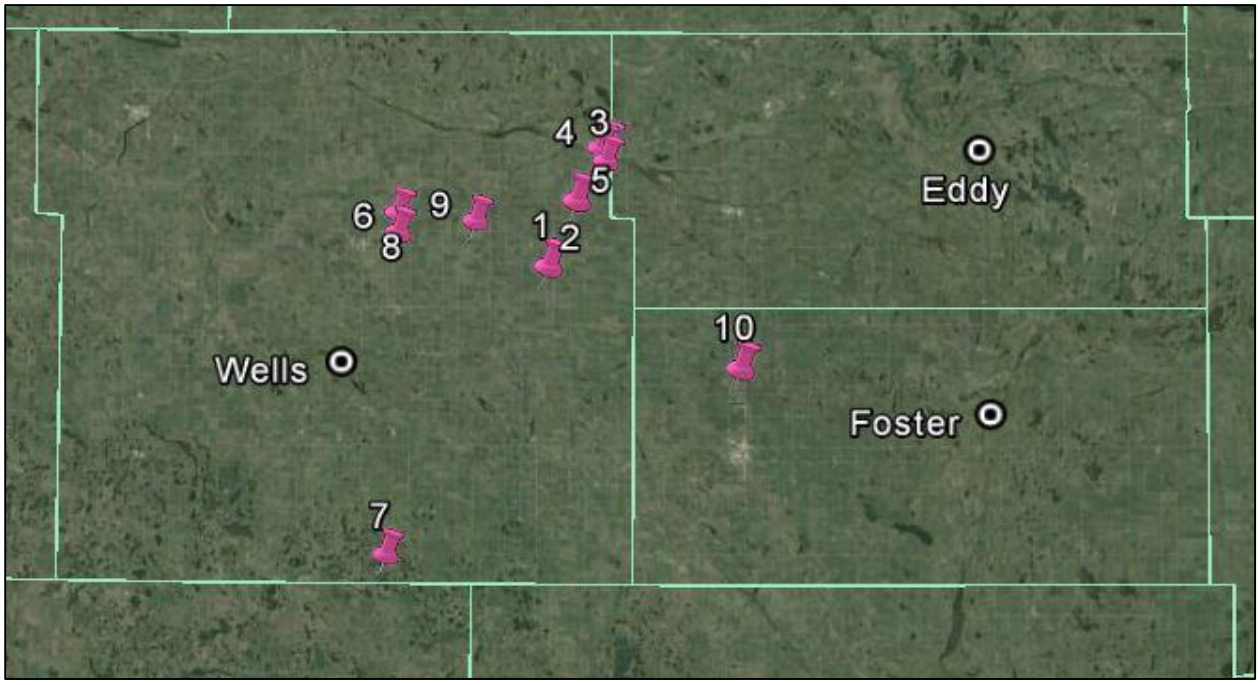

Figure 1.1. Map using the GPS coordinates of the fields where the 2012 anthracnose samples were collected.

Preliminary media analysis. Previous research indicated that depending on race, sporulation of *C. lindemuthianum* may be sparse and erratic on media (Mathur et al., 1950). Prior to race-typing, a study was performed to compare M3 media (Pinto et al., 2012) and Mathur's agar (Balardin et al., 1997) influences of *in vitro* sporulation of *C. lindemuthianum*. Agar plugs containing mycelia and conidia from actively growing cultures of all isolates of the *C. lindemuthianum* collected from field samples were placed on the agar surface of both media types and allowed to grow for 7 days in the dark at room temperature. The agar surface was scraped using 3 ml sterile distilled water and a sterilized bent glass rod. The solution was diluted 1:10 in sterile distilled water and conidia were counted using a hemocytometer. Data from the preliminary media analysis was analyzed using SAS version 9.3 (SAS Institute, Inc., Cary, NC).

Conidial production on the two types of media did not differ significantly; therefore, it was decided to use M3 media for further experiments.

Differential inoculation and race identification. Inoculum of three isolates from each field and all eight cultures from the CREC from 2012 was produced by transferring agar plugs containing mycelium and conidia to M3 media. A reference culture of race 73 was also produced by transferring an agar plug containing mycelium and conidia to M3 media. The cultures were incubated at room temperature in the dark for 7 to 14 days until profuse sporulation was present on the agar surface.

Pathogen races were determined by screening isolates against a standard set of 12 differentials with diverse origin and with one or more of the 13 known major resistance genes (Table 1.2). Seed from the set of differentials and one known susceptible cultivar, Lariat, were germinated on water agar for one week, planted using a complete randomized design in trays (25 cm × 50 cm) containing Sunshine Soil Mix No. 1 (Sun-Gro Horticulture, Canada), and allowed to grow until the primary leaves were fully expanded (V1). Three plants per differential were inoculated with a suspension containing 10^6 conidia/ml until run-off, placed in a humidity chamber for 5 days at 20°C under 14 h of fluorescent light, and returned to the greenhouse. Ten days after inoculation, disease reaction was measured based on a 1 to 9 severity scale (Balardin et al., 1997). Scores of 1 through 3 were recorded as resistant. These plants had no visible disease symptoms or only a few, very small lesions, mostly on the primary leaf veins. Scores of 4 through 9 were recorded as susceptible. Plants displayed numerous enlarged lesions or sunken cankers on the lower sides of leaves or hypocotyls (Balardin et al., 1997). A binary system was used to identify races of *C. lindemuthianum*. Binary numbers were according to Melotto et al. (2000). The binary number was equal to 2^n, where n is equal to the place of the differential

within the series. The additive sum of the binary numbers assigned to each differential with a susceptible reaction determined the specific *C. lindemuthianum* race (Table 1.2; Pastor-Corrales, 1991). For example, susceptible reactions on differentials 'Michelite,' 'Cornell 49242,' and 'Mexico 222' indicated the presence of race 73. Inoculations were performed twice for each isolate used.

Results

Pathogen isolations and race identification. In 2012, 90 isolates of *C. lindemuthianum* recovered using hyphal-tip methods were obtained from infected plant tissues from nine dry bean fields in Wells County and eight single-spore isolates were provided by Dr. Michael Wunsch collected from samples submitted to the CREC (Table 1.1). No anthracnose was reported in North Dakota in 2013. Of the 33 isolates from 2012 evaluated for race identification, three isolates were determined to be race 9, three isolates were determined to be race 72, and the remaining 27 isolates were determined to be race 73 based on reaction to the differential set and compared to the reaction of the isolate previously identified as race 73 (Table 1.1; Table 1.2).

Table 1.1. Number of *C. lindemuthianum* isolates obtained from fields in North Dakota in 2012 and the races identified.

County	Field no.	No. of isolates obtained	No. of isolates tested	Races identified
Wells	1	13	3	9, 73
Wells	2	10	3	9, 72, 73
Wells	3	13	3	73
Wells	4	2	2	73
Wells	5	24	3	73
Wells	6	8	3	9, 73
Wells	7	13	3	73
Wells	8	5	3	72, 73
Wells	9	2	2	73
Foster	10	8	8	72, 73

Table 1.2. Twelve differential lines used for race identification with the corresponding binary number and resistance genes in each line.

Differential	Binary no. [a]	Resistance gene [b]
Michelite	1	*Co-11*
Michigan Dark Red Kidney	2	*Co-1*
Perry Marrow	4	*Co-1*^{3}
Cornell 49242	8	*Co-2*
Widusa	16	*Co-1*^{5}, *Co-3*^{3}
Kaboon	32	*Co-1*^{2}
Mexico 222	64	*Co-3*
PI 207262	128	*Co-3*^{3}, *Co-4*^{3}
TO	256	*Co-4*
TU	512	*Co-5*
AB 136	1024	*Co-6*, *Co-8*
G 2333	2048	*Co-4*^{2}, *Co-5*, *Co-7*

[a] Binary numbers are according to Melotto et al. (2000). Binary number is equal to 2^n, where n is equal to the place of the cultivar within the series.
[b] Resistance genes are according to the gene list posted on the website for the Bean Improvement Cooperative (http://bic.css.msu.edu/_pdf/Bean_Genes_List_2014.pdf).

Discussion

Anthracnose infected pinto bean plants were found in nine fields in Wells County, North Dakota in 2012. In 2012, there were many nights in which the temperatures reached the dew point making the environment favorable for the development of inoculum of this pathogen (2012 NDAWN Monthly Report). The absence of anthracnose in 2013 could have been due to the unfavorable environmental conditions during the season. The summer of 2013 was very hot and dry, and anthracnose is favored by cool, humid conditions. It is also possible that anthracnose was present in 2013 but these fields were not identified.

Since anthracnose is known to be a seed transmitted disease, the use of certified clean seed is one of the most important management strategies available to growers. The North Dakota State Seed Department enforces a zero-tolerance (any level of *C. lindemuthianum* detected results in seed-lot rejection) policy for dry bean seed infected with *C. lindemuthianum* (NDSSD, 2008). The North Dakota State Seed Department has taken measures to ensure dry

bean seed produced in North Dakota is free from the pathogen. In 2002, the state began requiring certified seed grown in North Dakota to be tested for anthracnose. In addition to this, any imported seed is required to be tested for the presence of the disease. In 2002, a sample of pink bean seed imported from a seed lot from Manitoba, Canada, was discovered to be infected with race 1161 (Goswami et al., 2011). This further supports the importance of planting certified clean seed.

Overall, the assessment of the *C. lindemuthianum* isolates collected from infected fields indicates that race 73 is the predominant pathogen race in North Dakota. Cultivar 'Lariat' was the most commonly planted pinto bean in this area and is known to be susceptible to race 73 (Goswami et al., 2011; Knodel et al., 2014). In a previous study, 12 commercial dry bean cultivars (T-39, Matterhorn, Montcalm, Avalanche, Navigator, Norstar, Vista, Lariat, Maverick, Othello, Stampede, and Sedona) were evaluated for resistance against previously identified and new races of anthracnose, including 7, 73, 89, 1153, and 1161 (Goswami et al., 2011). Of these cultivars, all except the dark red kidney bean cultivar 'Montcalm', of Andean origin, were highly susceptible to race 73. The 2012 and 2013 Dry Bean Grower Surveys reported a low percentage of kidney bean acres in North Dakota during these years; hence the resistance offered by the 'Montcalm' kidney bean cultivar was not an option for growers (Knodel et al., 2013; 2014). The Mesoamerican differentials 'Michelite,' 'Cornell 49-242,' and 'Mexico 222' displayed a susceptible phenotype when inoculated with 2012 *C. lindemuthianum* isolates which were determined to be races 9, 72, and 73. The reaction on these differentials indicated that these races were of Mesoamerican origin, demonstrating host-pathogen coevolution resulting from selection pressure exerted by the plant and the pathogen (Balardin et al., 1997; Balardin and Kelly, 1998; Melotto et al., 2000; Pastor-Corrales et al, 1995; Young and Kelly, 1996).

Even though race 73 continues to be the most common race of *C. lindemuthianum* in North Dakota, it is important to keep in mind that other races, including 7, 9, 72, 89, 1153, and 1161, have been found. Races 9 and 72 found during the 2012 survey pose no threat to the dry bean industry in North Dakota as these races are virulent on fewer resistance genes than race 73. Races 89, 1153, and 1161 together accounted for less than 5% of the total detected races during the survey conducted between the years of 2003 and 2009 (Goswami et al., 2011). The low detection incidence could be why these races were not found during the survey conducted in 2012. It is important to remember that even though race 105 has not been found in North Dakota, it has been found very nearby in Manitoba, Canada. This race has a similar virulence pattern as race 73 except that it is also virulent on 'Kaboon' which contains *Co-1*^{2}. Introduction of race 105 to North Dakota could mean cultivars and breeding germplasm could be more susceptible. All of these races present in North Dakota have the capability of infecting dry beans with varying levels of disease severity; however, there are sources of resistance available to be used for breeding dry bean lines suitable for this region. In a recent study, possible resistance genes that could be effective against races 73 and 105 include *Co-1*, *Co-1*^{5}, *Co-4*, *Co-4*^{2}, and *Co-5* (Dongfang et al., 2008). *Co-4*^{2}, which encodes a protein kinase (Melotto and Kelly, 2001), is of special interest because research has shown that this gene confers resistance to 97 percent of the *C. lindemuthianum* races present in North and South America (Balardin and Kelly, 1998). Further research is needed to evaluate these genes and others available for resistance to the pathogen races. The ongoing evaluation of pathogen races is paramount to breeding for resistance to *C. lindemuthianum* and providing growers with resistant cultivars.

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CHAPTER 2. SEED TO SEEDLING TRANSMISSION OF *COLLETOTRICHUM LINDEMUTHIANUM* IN STEM TISSUE UNDER GREENHOUSE AND FIELD CONDITIONS

Introduction

Anthracnose of dry bean (*Phaseolus vulgaris* L.), caused by the fungal pathogen *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib., can be a damaging disease causing large reductions in yield and reduced seed quality. Its teleomorph, *Glomerella lindemuthiana* (Shear), is rarely found in nature (Pastor-Corrales and Tu, 1989). Anthracnose is primarily seed-borne and affects all aerial parts of the plant. Symptomatic plants display dark brown-black, slender lesions that follow the vein on the abaxial side of the leaf. Lesions on petioles and branches are oval-shaped while lesions on pods are circular and sunken with raised edges (Schwartz et al., 2005). When the lesions are wet for a period of time, a pale, salmon-colored gel-like fluid containing masses of conidia develops from the acervuli (Schwartz et al., 2005).

Planting disease-free bean seed is the most effective option available to manage anthracnose in commercial fields. The North Dakota State Seed Department has taken measures to ensure dry bean seed produced in North Dakota is free from the pathogen by enforcing a zero-tolerance (any level of *C. lindemuthianum* detected results in seed-lot rejection) policy for dry bean seed infected with *C. lindemuthianum* (NDSSD, 2008). In 2002, the state began requiring certified seed grown in North Dakota to be tested for anthracnose. The use of certified, clean seed from fields that have been field inspected and tested post-harvest for this pathogen is recommended. Planting bin run seed should be avoided.

Traditionally, anthracnose of dry bean has been diagnosed via pathogen isolation from infected tissues using nutrient agar. Visual detection of morphological structures diagnostic of *C. lindemuthianum* can take 14 or more days to complete (International Seed Testing Association, 2008). Distinguishing anthracnose from other diseases that have similar symptoms particularly on seed, such has common bacterial blight or halo blight or abiotic sunscald, can be difficult without confirmation of fungal growth. These methods are labor intensive and may not be sensitive enough to detect symptomless seed infections. If the pathogen is not visually detected, the tissue sample or seed may be deemed healthy.

A PCR-based system was developed for the detection of *C. lindemuthianum* in symptomatic and asymptomatic seeds and infected plant tissue using nested PCR (Chen et al., 2007). Forward primer ClF4 was designed from the ITS1 region and used with universal primer ITS4 to produce an amplicon specific to *C. lindemuthianum* (Chen et al., 2007). Soon after, another PCR-based system was developed (Wang et al., 2008). Two primer pairs (CY1/CY2 and CD1/CD2) were used in a duplex conventional PCR assay. These primer pairs produced products that were 442 and 638 base pairs, respectively, that were unique to *C. lindemuthianum*. The duplex PCR assay was developed to separate *C. orbiculae*, the causal agent of anthracnose on cucurbits, from *C. lindemuthianum* due to the similarities of the ITS sequences of these two organisms (Wang et al., 2008). Later research resulted in the development of a real-time qPCR-based assay utilizing SYBR green dye and primer pair ClF432/ClR533 that resulted in the detection of *C. lindemuthianum* in bean tissue (Chen et al., 2013). A TaqMan probe assay utilizing the same primers and probe ClP442 produced similar results (Chen et al., 2013). This primer set was utilized to study the relationship between the level of symptoms on bean seed, including asymptomatic, and the amount of pathogen DNA in bean seed. Results indicated that

the lesion size on symptomatic seed strongly correlated with the amount of pathogen DNA detected by the real-time qPCR assay. However, only a weak fluorescent signal was detected in asymptomatic bean seed (Chen et al., 2013). No clear indication was provided to determine if the weak fluorescent signal from asymptomatic seed could be clearly distinguished from background fluorescence or healthy seed; therefore, a more reliable real-time qPCR assay is needed.

Real-time qPCR assays can provide an efficient and effective diagnostic tool for the evaluation of host-pathogen interactions. Real-time qPCR assays allow detection of *C. lindemuthianum* to be completed within two to three days instead of the laborious 10 to 14 days needed to complete a traditional detection assay. Additionally, real-time qPCR has the ability to detect symptomless infections at a higher frequency than that observed with traditional evaluations, and misidentification occurs much less frequently (McCartney et al., 2003). Molecular detection can also provide insights into pathogen biology and etiology by allowing studies to be done detailing the interaction between the host and the pathogen.

Previously conducted research has demonstrated an increase in seedling infection with increasing severity of seed infection (Conner et al., 2009; Tu, 1983). Also, previous studies conducted to evaluate seed to seedling transmission were based only on symptom expression, and did not evaluate the presence of the pathogen (Conner et al., 2006; 2009; Mohammed and Sangchote, 2005; 2007). While it is known that *C. lindemuthianum* moves from infected seed into the seedling to cause sometimes devastating levels of disease, to date, no research has been performed to quantify the pathogen within host tissue. The research proposed here will aid in our understanding of seed to seedling transmission of the pathogen. The objectives of this research were to evaluate the utility of real-time qPCR assays to quantify the pathogen in stem

tissue produced from infected seed and to compare traditional methods of detection of *C. lindemuthianum* in dry edible pinto bean seed, including symptomless seed infections, to new and existing real-time qPCR assays. These objectives were accomplished by developing a real-time qPCR assay, quantifying *C. lindemuthianum* in symptomatic and asymptomatic dry bean seed, and evaluating seed germination, seedling emergence, visual assessment of disease symptoms, and pathogen quantification in stem tissue under greenhouse and field conditions.

Materials and methods

C. lindemuthianum isolate preparation and maintenance. *C. lindemuthianum* isolates collected from a disease survey conducted during the 2012 growing season originating from pinto bean plants produced in North Dakota were used to develop, perform, and validate real-time qPCR assays. Dry bean plants exhibiting anthracnose symptoms were surface disinfested and purified as described in Chapter 1. Isolates were preserved in long-term storage via transfer onto PDA overlaid with one layer of sterilized Whatman number 1 filter paper for approximately 2 to 3 weeks or until the filter paper was completely colonized. The filter paper was removed, placed in an empty Petri dish, and incubated in the dark at room temperature until the paper and fungus were completely dried, approximately 20 to 30 days. The dried filter paper was aseptically transferred into a sterile 15 ml centrifuge tube and stored at 4°C. Isolates were also preserved by putting 10 plugs of PDA containing conidia and mycelium of *C. lindemuthianum* in a 2 ml cryogenic vial with 30% glycerol and stored at -80°C.

Seed collection and sorting. For all seed evaluations and greenhouse and field trials, healthy seed from non-infected 'AC Pintoba' (Park et al., 2003) was obtained from the Morden Research Station, Agriculture and Agri-Food Canada, Morden, Manitoba, Canada or from greenhouse-reared plants. Infected 'AC Pintoba' seed with varying degrees of symptom severity

was acquired from Dr. Michael Wunsch at the Carrington Research and Experimental Center (CREC) or was collected from plants inoculated with *C. lindemuthianum* race 73 grown under greenhouse conditions. Seed obtained from the healthy source was considered as such and the seed from the infected seed source was placed into five categories based on the lack of or the presence of visual symptoms of anthracnose. For the purpose of this project, a total of six seed categories were used - 1) healthy, 2) symptomless, 3) slightly discolored, 4) discolored, 5) lesions covering less than 50% of one side of the seed coat, and 6) severely infected (Fig. 2.1).

Figure 2.1. Disease categories of infected seed ranging from symptomless to severe infection. Seed categories are as follows: 1) healthy, 2) symptomless, 3) slightly discolored, 4) discolored, 5) lesions covering less than 50% of one side of the seed coat, and 6) severely infected.

Examination of anthracnose symptomatic and asymptomatic seeds. Anthracnose symptomatic and asymptomatic seed and healthy seed of the dry pinto bean cultivar 'AC Pintoba' was evaluated for *C. lindemuthianum* using the International Seed Testing Association

(ISTA) validated methods (ISTA, 2008). One hundred fifty seeds each of the six seed categories (Fig. 2.1) were placed in a tea strainer, surface disinfested in a 10% solution of sodium hypochlorite for 10 min and the excess solution was allowed to drain. The seed was spread on double sheets of wet paper towels; three replicates of 50 seeds were wrapped in plastic wrap to maintain moisture and incubated for 7 days at 20°C in the dark. Seed coats were removed and the cotyledons were examined for characteristics of anthracnose and signs of *C. lindemuthianum* growth.

Greenhouse trials. Seed for greenhouse assays were obtained from the sources described above. Fifty seeds from the seed categories of 1) healthy, 2) symptomless, 3) slightly discolored, and 4) discolored and 100 seeds from the seed categories of 5) lesions covering less than 50% of one side of the seed coat and 6) severely infected (Fig. 2.1) were surface disinfested as described above, and placed on solid media in a Petri plate to allow the seed to germinate for 7 days in a 20°C incubator. Twice as many seeds from the seed categories 5 and 6 were used to try to ensure enough germinated seed for planting. Percent germination was determined and 40 germinated seeds from each seed category were planted in a randomized complete block design (RCBD) with 4 replicates in 6 in. red clay pots filled with Sunshine Soil Mix No. 1 (Sun-Gro Horticulture, Canada). Emergence was recorded 10 days after planting (DAP).

Emerged plants were humidified using an amended plant misting protocol (Tu, 1983). Plants were misted with tap water until run-off, covered with black plastic bags for 72 h at 21°C, uncovered, and incubated in a greenhouse room with a 14 h light period for 4 days. This process began 10 DAP and was repeated every 7 days for 4 weeks. Symptoms of anthracnose disease were recorded prior to each time plants were covered. Two plants from each category/replicate were sampled every 7 days for 5 weeks beginning 10 DAP (the first sample was taken before

humidification). Plant height, root length, fresh plant and root weight were recorded immediately after harvest. A segment of each stem approximately 2.5 cm above the soil line and below the cotyledon was placed in individually labeled small Ziploc bags. Stem segments were washed once in a 10% sodium hypochlorite solution for one minute followed by three rinses in sterile distilled water and allowed to dry until the excess water evaporated. Ten discs of stem tissue, weighing approximately 100 mg total, were excised from the basal region of the stem segment. These 10 discs were cut in half and 10 disc halves were placed in a small plastic bag. Sterile distilled water was added at a 1:2 (wt/vol) ratio. Stem segments were then crushed and 50 µl was spread onto two Petri dishes containing solid PDA using a sterilized bent glass rod. The visual presence or absence of *C. lindemuthianum* was recorded after incubation in the dark at room temperature for one week. The other 10 disc halves were stored at 4°C in XXTuff 2 ml microvials (BioSpec Products) with 3 to 5-2.3 mm chrome steel beads for DNA extraction.

Field trials. During the 2013 growing season, a field trial was conducted at a location two miles southeast of Sykeston, ND, whereas during the 2014 growing season, a field trial was conducted at the Morden Research Station in Morden, Manitoba, Canada. 'AC Pintoba' pinto bean seeds were obtained from the sources described above and sorted into seed categories. Only seed categories 1 through 4 were used because an insufficient number of seeds in categories 5 and 6 were identified and germination of seeds in these categories was low based on previous studies. The trials were planted in a RCBD with six replicates. Each plot consisted of 4-9.1 m rows, with 0.3 m spacing between rows. Planting was based on approximately 270,000 plants per hectare. For the 2013 trial, buffer and guard plots were seeded to healthy 'Lariat' pinto beans. Soybeans, a non-host of *C. lindemuthianum*, were used as a buffer during the 2014 trial.

In 2013, the trial was planted on June 12, emergence was determined 15 and 21 DAP, and plots were scouted for symptoms prior to sampling. Ten plants from the end of each of the four rows per treatment/replicate were carefully lifted from the soil as to not disrupt the root system starting 15 DAP and continuing for 3 weeks. Fresh plant weight, length, and disease severity were obtained from each plant within 2 hours of harvest. Disease severity per plant was assessed by visually estimating the percent of above ground plant tissue affected by the disease. A segment of each stem approximately 2.5 cm above the soil line and below the cotyledon was placed in individually labeled small Ziploc bags. Stem segments were brought back to the research lab, processed and stored as previously described for the greenhouse trials. Because of poor emergence, the field trial was destroyed after all stem segments had been collected and prior to crop maturity.

In 2014, the trial was planted on May 23, and the same procedure was followed except emergence was determined 10 and 17 DAP and the plants were lifted from the soil beginning 10 DAP. A segment of each stem approximately 2.5 cm above the soil line and below the cotyledon was placed in individually labeled small manila envelopes and laid out to dry at the Morden research lab for one week. Stem segments were mailed back the NDSU research lab and processed for a plating assay and real-time qPCR. The segments were placed in a 2 ml centrifuge tube, lyophilized for 24 hours, and stored at -80°C until *C. lindemuthianum* quantification was performed. The lyophilized stem tissue was shredded; 35 to 40 mg of the tissue was placed in an XXTuff 2 ml microvial with 3 to 5-2.3 mm chrome steel beads, and ground in a VWR high throughput homogenizer until a fine powder consistency was reached. Two quantities of 10 mg each of the stem powder were spread onto two Petri dishes containing

solid PDA. Visual growth of *C. lindemuthianum* was recorded after incubation in the dark at room temperature for one week.

After sample collections were completed, disease severity was assessed 46, 61, 75, and 82 DAP by visually estimating the percentage of leaf veins, stems, and pods affected by anthracnose at four random sites within each plot. Plants were allowed to reach full maturity, and the trial was harvested on September 16. Harvested seed was evaluated for anthracnose symptoms. The harvest weight included all beans over a #6 round screen which aspirated any chaff (small soil lumps were handpicked out); all splits and small bean seeds were included. Two hundred seeds were shaken over a #10 slotted screen to remove splits and small seeds and evaluated for anthracnose symptoms of discoloration or lesions.

Extraction of total genomic DNA from fungal tissue, seed, and stem tissue. Total genomic DNA was extracted from mycelium of *C. lindemuthianum* isolates grown in 50 ml of Richard Solution (10 g sucrose, 10 g KNO_3, 5 g KH_2PO_4, 1.22 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeCl_3$, 150 ml V8 juice, 850 ml H_2O) (Tuite, 1969) for 7 to 10 days under constant shaking at 22± 2°C. Media was removed via vacuum filtration, fungal material was lyophilized for 24 hours, and DNA was extracted using a CTAB DNA extraction protocol (Stewart and Via, 1999).

Seeds to be tested using real-time qPCR were placed in a 4 ml vial with a 9.5 mm steel ball bearing and ground in a VWR high throughput homogenizer set at speed 10 for 3 min. This was repeated until the seeds were reduced to flour. Fifty milligrams of the flour from each seed was used for DNA extraction. DNA extraction was performed on the ten stem halves stored from the fresh greenhouse and 2013 field stem samples by grinding in an XXTuff 2 ml microvial with 3 to 5-2.3 mm chrome steel beads (BioSpec Products) in a VWR high throughput homogenizer at speed 10 for 3 min. For the 2014 field stem samples, 10 mg of powder was used

to extract DNA. DNA from fresh and dried stem tissue and seed was extracted using the DNeasy Plant Mini Kit (Qiagen Inc.) according to manufacturer's protocol. Fifty microliters of elution buffer AE from the kit was used to elute the genomic DNA from field stem tissue. Two hundred microliters of elution buffer AE from the kit was used to elute genomic DNA from seed and greenhouse stem tissue. All DNA was quantified individually using the NanoDrop 2000 and used as the template for real-time qPCR assays.

Primer pair development and validation. Real-time qPCR primers were developed from essential genomic regions of *C. lindemuthianum* for quantification of pathogen DNA in dry bean plant tissue and seed. Eleven pairs of forward and reverse primers were designed using Primer3 from the National Center for Biotechnology Information (NCBI) GenBank database (Table 2.1). Primer pairs with a 3' complementarity score closest to zero where chosen for optimization. Each primer pair had a unique melting temperature. These theoretical melting temperatures were used as a starting point to develop a temperature gradient, ranging from 52°C to 60°C, to determine the optimal practical temperature. Reactions were performed using 20 µl total volumes consisting of 1x SsoAdvanced Universal SYBR Green SuperMix (Bio-Rad Laboratories), 500 nM of each primer (Integrated DNA Technologies), and 2 µl of template DNA in the Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories). PCR-grade water was added to reach the final reaction volume. Sensitivity of the primer sets was evaluated using two trials of real-time qPCR performed on six 10-fold serial dilutions of DNA extracted from fungal cultures using duplicate reactions performed twice. Efficiency was calculated by using the formula: efficiency = $(10^{(-1/\text{slope})})-1$. All reactions were run using a program of 95°C for 2 min followed by 40 cycles of 95°C for 5 sec and 30 sec using the temperature gradient between 52°C

to 60°C, and a melt curve phase of 1 cycle at 65°C to 95°C (ramp speed 0.5°C/0.5 s) was programmed at the end.

Table 2.1. Real-time PCR primers designed for quantification of *Colletotrichum lindemuthianum* and the respective target genes.

Primer pair	Gene target
ClF1527/ClR1609 [a]	Nitrate reductase [b]
ClF1528/ClR1641	Nitrate reductase [b]
ClF2640/ClR2742	Nitrate reductase [b]
ClF1238/ClR1368	Pectin lyase [c]
ClF420/ClR503	Pectin lyase [c]
ClF360/ClR446	18S, 5.8S, & 28s rRNA (located in ITS regions 1 & 2) [d]
ClF361/ClR571	18S, 5.8S, & 28s rRNA (located in ITS regions 1 & 2) [d]
ClF621/ClR712	Histone kinase [e]
ClF3391/ClR3507	Histone kinase [e]
ClF183/ClR293	Pac protein [f]
ClF929/ClR1127	Pac protein [f]

[a] indicates the primer pair chosen for further evaluations.
[b] GenBank accession number JF681041.1
[c] GenBank accession number JX270683
[d] GenBank accession number JN198431.1
[e] GenBank accession number GU907087.1
[f] GenBank accession number JX679084.1.

Evaluations using primer pair ClF1527/ClR1609. Primer concentrations were evaluated using DNA extracted from stem tissue grown under greenhouse conditions. Final reactions consisted of 1x SsoAdvanced Universal SYBR Green SuperMix, 200 nM of each primer, and 2 µl of template DNA in the Bio-Rad C1000 Touch Thermal Cycler. PCR-grade water was added to adjust the final reaction volume. Real-time qPCR conditions were 2 min at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 57°C. A Cq value of 33.00 or above was considered negative, or no fungal detection. Sensitivity of the new real-time qPCR assay *in planta* was evaluated using DNA extracted from 7 replicates of 5 seeds from each of the 6 seed categories. To test assay specificity, selected amplicons were sequenced. Additionally, specificity of the primer set was evaluated using real-time qPCR performed against numerous fungal and bacterial

pathogens of dry beans from the NDSU Dry Bean and Pulse Pathology laboratory collection and several *Colletotrichum* species DNA samples provided by Dr. S. Banniza, University of Saskatchewan, and Dr. L. Vaillancourt, University of Kentucky (Table 2.3).

In planta quantification of *C. lindemuthianum*. The real-time qPCR assay using the new primer pair ClF1527/ClR1609 was utilized to quantify *C. lindemuthianum* in stem and seed tissue generated in field and greenhouse trials. Fungal quantity was based on a standard curve of ten-fold serial dilutions of *C. lindemuthianum* DNA. DNA from stem samples collected across sampling dates from the greenhouse and field trials as well as seed from planting sources was evaluated using assay parameters described above.

Comparison of *C. lindemuthianum in planta* quantification using four SYBR Green assays. In total, four real-time qPCR assays consisting of primer pair ClF1527/ClR1609 with and without the probe ClP1563 and primer pair ClF432/ClR533 with and without the probe ClP442 were compared. All four assays were tested on a set of 174 stem tissue DNA samples grown in the greenhouse trials. The previously developed real-time qPCR assay using primer pair ClF432/ClR533 (Chen et al., 2013) was compared to ClF1527/ClR1609 using DNA extracted from seed of all six symptom severity categories. All reactions using ClF432/ClR533 were performed as previously described with slight modifications and consisted of 1x SsoAdvanced Universal SYBR Green SuperMix, 500 nM of each primer, 2 µl of template DNA in the Bio-Rad C1000 Touch Thermal Cycler, and PCR-grade water was added to adjust the final reaction volume to 20 µl (Chen et al., 2013). Reactions using ClP442 were also performed as previously described with slight modifications and consisted of 1x SsoAdvanced Universal Probes SuperMix, 500 nM of each primer, 100 nM of the probe, and 2 µl of template DNA (Chen et al., 2013). Cq thresholds were as reported (Chen et al., 2013).

Using the product sequence of the new primer pair ClF1527/ClR1609 and Primer3, a probe was developed to be used in conjunction with the new primer pair (Table 2.2). Reactions using the probe ClP1563 consisted of 1x SsoAdvanced Universal Probes SuperMix, 200 nM of each primer, 100 nM of the probe (Integrated DNA Technologies), and 2 µl of template DNA in the Bio-Rad C1000 Touch Thermal Cycler. Real-time qPCR conditions and Cq threshold were the same as previously described for ClF1527/ClR1609 alone.

Table 2.2. Sequences of the final primer pair ClF1527/ClR1609 and the probe CP1563 used for the detection of *C. lindemuthianum.*

Primer/ Probe	**Sense**	**Sequence (5'-3')**	**Amplicon size**
ClF1527[a]	Forward	CAATCTCACCAACGGCTTCT	83 bp
ClR1609[a]	Reverse	AAATCTCCCTGAACGGCTCT	…
ClP1563[a]	Probe	56-FAM/AGGAGAGAGCGAAAACACCGTC/3BHQ_1	…

[a] GenBank accession number JF681041.1.

The correlations of the Cq values were used to compare the sensitivities of the real-time qPCR assays. Four quadrants were developed on the correlation graphs using the Cq value thresholds for each assay. The Cq thresholds for ClF432/ClR533 and ClP442 were determined as recommended by Chen et al. (2013), and the threshold for ClF1527/ClR1609 and ClP1563 were used as determined by this project. Quadrant I was comprised of those samples tested positive by both assays, quadrant II – samples tested positive only by the assay on the y-axis, quadrant III – samples tested negative by both assays, and quadrant IV – samples tested positive only by the assay on the x-axis (Fig. 2.2).

Statistical analyses. Analyses of variance (ANOVA) were conducted using PROC GLM of SAS (version 9.3, SAS Institute, Cary, NC) for germination, emergence, plant heights and weights, root weights and lengths, *C. lindemuthianum* detection incidences and quantification, anthracnose severity, seed discoloration, and yield data collected from the field, greenhouse, and

real-time qPCR assays. *C. lindemuthianum* quantification did not meet the normal distribution of data assumption required by ANOVA; therefore, a $1+\log_{10}$ transformation was performed. Means were compared by Fisher's protected least significant difference test ($\alpha < 0.05$). Levene's test was used to verify homogeneity of variance (HOV) between trials and the interaction between the trial-treatment effects was evaluated where appropriate prior to data being combined for further analysis ($\alpha < 0.05$). Pearson's correlation analyses were used to calculate relationships between parameters in both field and greenhouse studies.

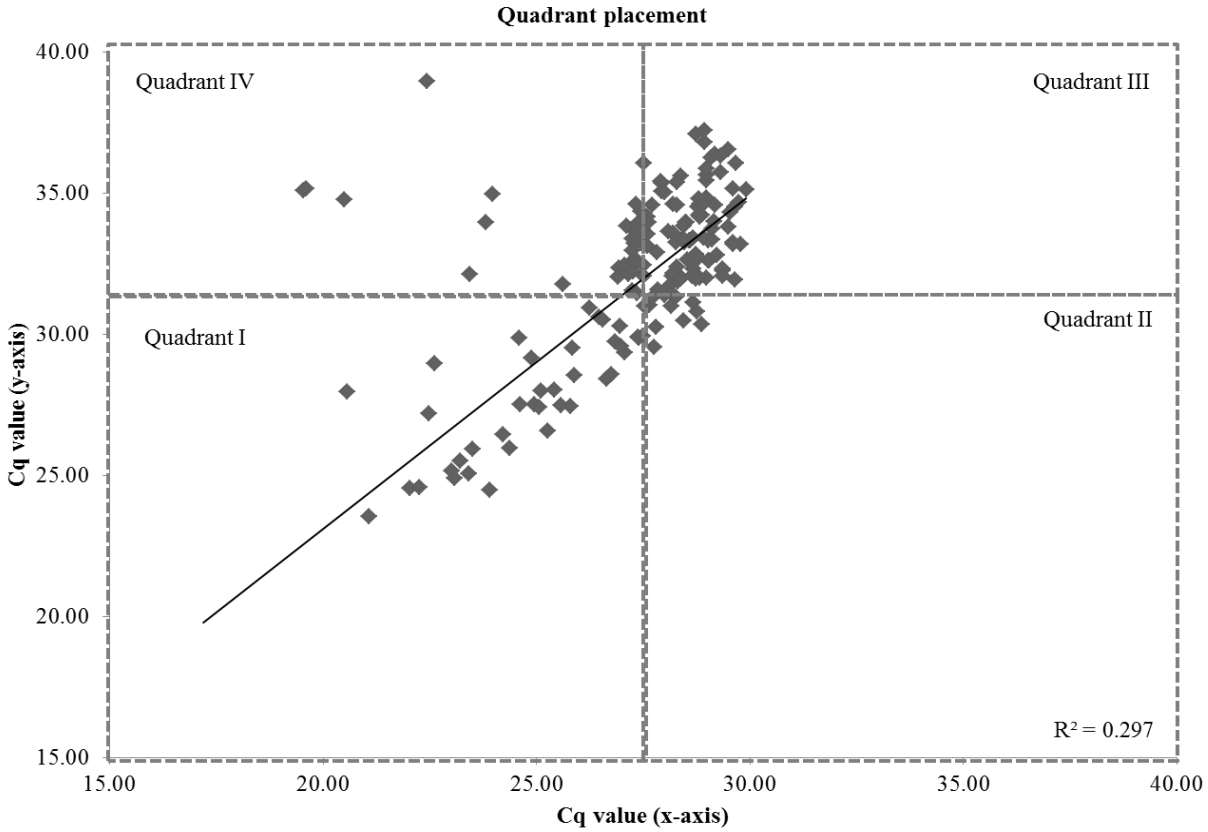

Figure 2.2. Example of quadrant placement on a correlation graph comparing two real-time qPCR assays when validating ClF1527/ClR1609. Quadrant I was comprised of those samples tested positive by both assays, quadrant II – samples tested positive by the assay on the y-axis, quadrant III – samples tested negative by both assays, and quadrant IV – samples tested positive by the assay on the x-axis. Quadrant line placement was determined by the Cq thresholds for each real-time qPCR assay.

Results

Development of real-time qPCR primers specific for *C. lindemuthianum.* Eleven forward and reverse primer pairs were developed for the detection of *C. lindemuthianum* in host tissue and seed. Based on reaction efficiency and sensitivity, primer pair ClF1527/ClR1609 was chosen for further optimization. Strong linear relationships between the Cq values and the log of the amount of fungal DNA were shown bya high correlation coefficient ($R^2 = 0.992$) and a slope of -3.3, indicating amplification efficiency of 100.1% (Table 2.3). This primer pair produced an amplicon that was unique to *C. lindemuthianum* and no amplicon was produced from any of the other *Colletotrichum* species or fungal or bacterial bean pathogens evaluated (Table 2.4).

Table 2.3. Amplicon sizes, regression coefficients, and efficiencies of primer pairs designed for the quantification of *Colletotrichum lindemuthianum* in real-time polymerase chain reactions.

Primer pair	Amplicon size (bp)	Regression coefficient (R^2)	Efficiency (%)[a]
ClF1527/ClR1609	83	0.992	100.1
ClF1528/ClR1641	114	0.981	104.0
ClF2640/ClR2742	103	0.972	115.1
ClF1238/ClR1368	131	0.991	99.5
ClF420/ClR503	84	0.995	99.4
ClF360/ClR446	87	0.938	147.1
ClF361/ClR571	157	0.833	411.5
ClF621/ClR712	92	0.974	121.6
ClF3391/ClR3507	117	0.866	116.3
ClF183/ClR293	111	0.973	94.3
ClF929/ClR1127	199	0.978	98.0

[a] Efficiency = $(10^{(-1/slope)})-1$

Table 2.4. Specificity of real-time polymerase chain reaction assays used for the quantification of *Colletotrichum lindemuthianum* using primer pair ClF1527/ClR1609.

Microorganism species	Dry bean disease	Host	+/- [a]
Colletotrichum lindemuthianum [e]	Anthracnose	Dry bean	+
C. acutatum [d]	…	Almond	-
C. acutatum [d]	…	Apple	-
C. acutatum [d]	…	Strawberry	-
C. cereale [d]	…	Orchardgrass	-
C. cereale [d]	…	Rye	-
C. coccodes	…	Potato	-
C. dematium [d]	…	Field eryngo	-
C. destructivum [c]	…	Red clover	-
C. graminicola [d]	…	Corn	-
C. higginsianum [c]	…	Bok choy	-
C. linicola [c]	…	Flax	-
C. sublineolum [d]	…	Sorghum	-
C. sublineolum [d]	…	Wheat	-
C. truncatum [c]	…	Lentil	-
C. truncatum [c]	…	Lima bean	-
C. truncatum [c]	…	Scentless chamomile	-
C. truncatum [c]	…	Soybean	-
Ascochyta lentis [e]	Ascochyta	Dry bean	-
Ascochyta rabiei [e]	Ascochyta	Dry bean	-
Curtobacterium flaccumfaciens pv. *flaccumfaciens* [e]	Bacterial wilt	Dry bean	-
Fusarium graminearum [e]	Fusarium root rot	Dry bean	-
Fusarium solani [e]	Fusarium root rot	Dry bean	-
Pseudomonas syringae pv. *phaseolicola* [e]	Halo blight	Dry bean	-
Pseudomonas syringae pv. *syringae* [e]	Brown spot	Dry bean	-
Rhizoctonia solani [e]	Rhizoctonia root rot	Dry bean	-
Sclerotinia sclerotiorum [e]	White mold	Dry bean	-
Uromyces phaseoli [e]	Bean rust	Dry bean	-
Xanthomonas spp. [b, e]	…	…	-
Xanthomonas axonopodis pv. *phaseoli* [e]	Common blight	…	-
Phaseolus vulgaris	…	…	-
PCR-grade water	…	…	-

[a] Positive or negative results denoted as + or -, respectively. Samples with Cq values greater than 33 are reported as negative.
[b] non-pathogenic species.
[c] DNA generously provided by S. Banniza, University of Saskatchewan.
[d] DNA generously provided by L. Vaillancourt, University of Kentucky.
[e] NDSU Dry Bean and Pulse Pathology laboratory.

Seed colonization increased as seed symptom severity increased. Although the differences were not significant, the real-time qPCR assay using ClF1527/ClR1609 was able to detect *C. lindemuthianum* in 17% of slightly discolored and discolored seeds produced from infected pods, compared to less than 11% detection incidence using the ISTA protocol (Fig. 2.3). The incidence of seed colonization by *C. lindemuthianum* in lesions <50% and severely infected seed was significantly higher than any other category using both methods. *C. lindemuthianum* detection incidence of discolored seed was significantly higher than symptomless only when the real-time qPCR assay was used for detection. No colonization was detected in healthy seed. Cq values for *C. lindemuthianum* DNA detected in positive seed ranged from 20.17 to 32.99; the lowest limit of this assay for detection of pathogen DNA in positive seed was 0.221 ng/µl based on the standard curve of 10-fold serial dilutions of *C. lindemuthianum* fungal DNA (Fig. 2.4). The level of colonization as determined using the real-time qPCR assay followed a similar trend as the detection incidence, colonization of severely infected seed was significantly higher than all other categories, but no significant difference was observed among the symptomless, slightly discolored and discolored seed.

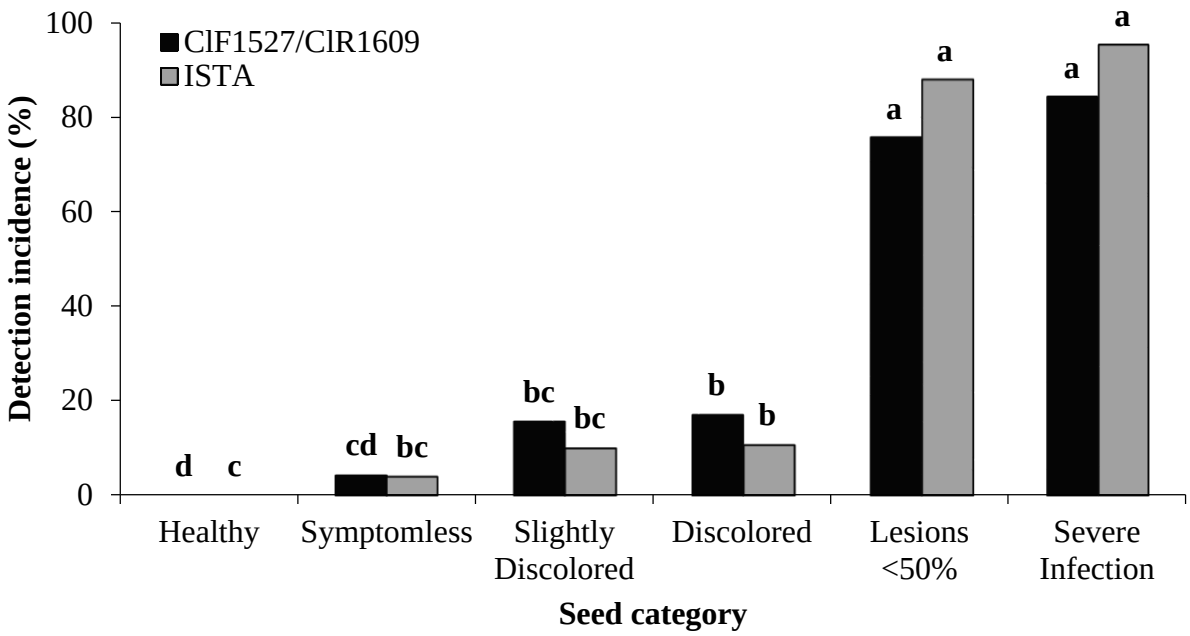

Figure 2.3. Detection (%) of *Colletotrichum lindemuthianum* in seed from six asymptomatic and symptomatic seed categories using real-time qPCR and ISTA methods. Columns labeled with the same letter compare the seed categories within method and are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

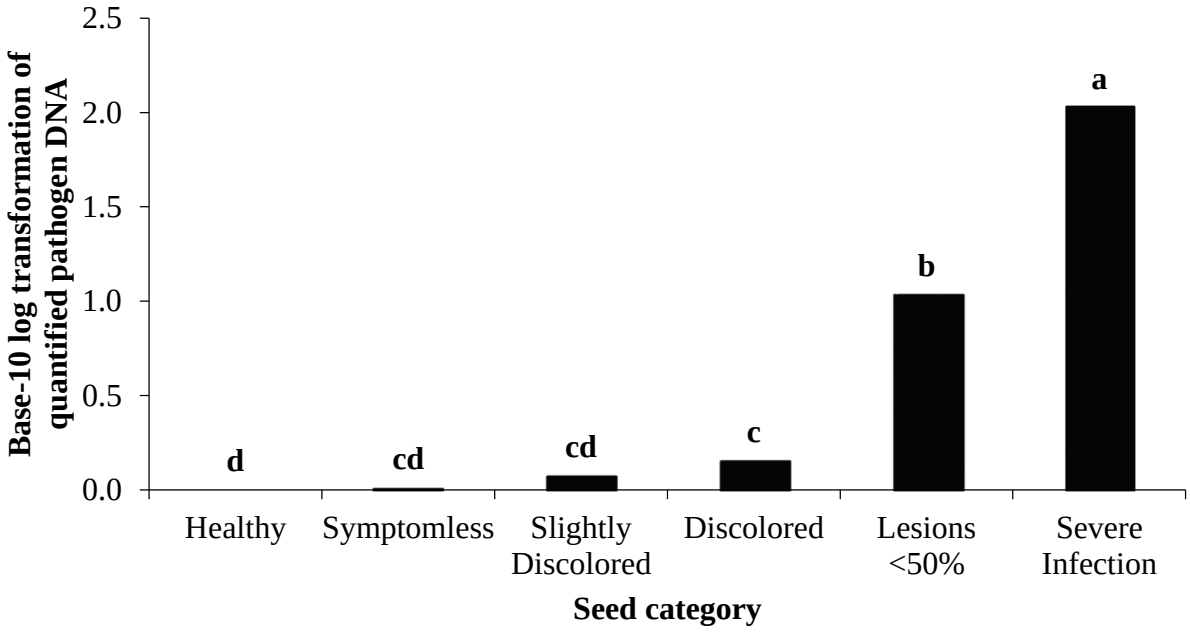

Figure 2.4. Level of pathogen colonization (ng/µl) across six asymptomatic and symptomatic seed categories determined by real-time qPCR assays using primer pair ClF1527/ClR1609. Columns labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

Evaluation of data from the greenhouse trials. Levene's test for HOV between trials was not significant for seed germination ($P = 0.9940$) or for seedling emergence ($P = 0.9675$). Additionally, no interaction between trials and seed categories was observed for seed germination ($P = 0.2255$) or for seedling emergence ($P = 0.3739$); therefore, data from the two trials were combined for further analyses. Results from the greenhouse experiments demonstrated significant decrease in seed germination and seedling emergence as symptoms of anthracnose on the seed increased (Fig. 2.5). Germination and emergence were highest in healthy seed. Germination was significantly higher in heathy seed than all other categories except symptomless seed. Emergence in healthy seed was only significantly higher than seed with lesions <50% and severe infection. Seeds with severe infection displayed significantly lower germination (35%) and emergence (29%) than seed in all other categories. Additionally, seed germination ($r = -0.9844$; $P = 0.0156$) and seedling emergence ($r = -0.9758$; $P = 0.0242$) were negatively and significantly correlated with the level of pathogen DNA detected in seed as determined by the real-time qPCR assay.

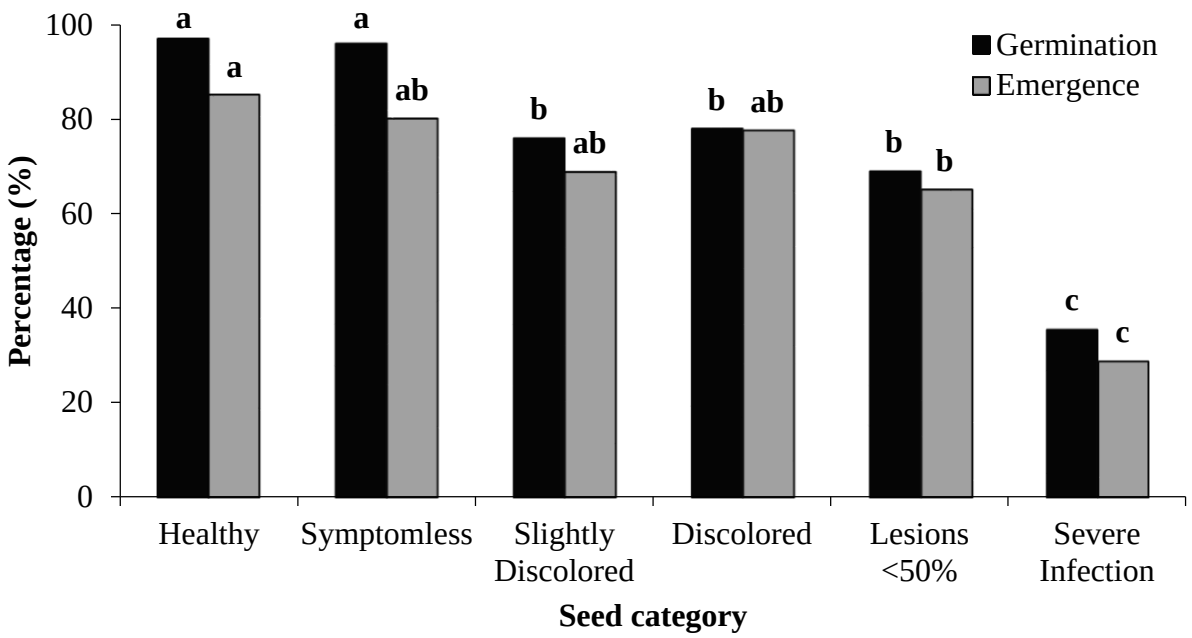

Figure 2.5. Combined analysis of seed germination (%) and seedling emergence (%) across six asymptomatic and symptomatic seed categories and two greenhouse trials. Columns labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

Levene's test for HOV between trial A and trial B for disease severity was not significant ($P = 0.5547$) and no interaction between trials and seed categories was observed ($P = 0.1493$); therefore, the data from the two trials were combined for further analyses. Disease severity for each trial displayed variability between sampling dates and seed categories (Fig. 2.6). Anthracnose severity 10 DAP consistently increased, sometimes significantly, as the level of seed symptom severity increased. However, this was not always true for data taken the following weeks, and overall disease severity did not increase with time. Weekly collection of samples and subsequent reduction in plants available to rate most likely played a role in this variability. The number of plants was reduced to eight and zero in the symptomless and severe infection seed categories, respectively, by the final data collection date.

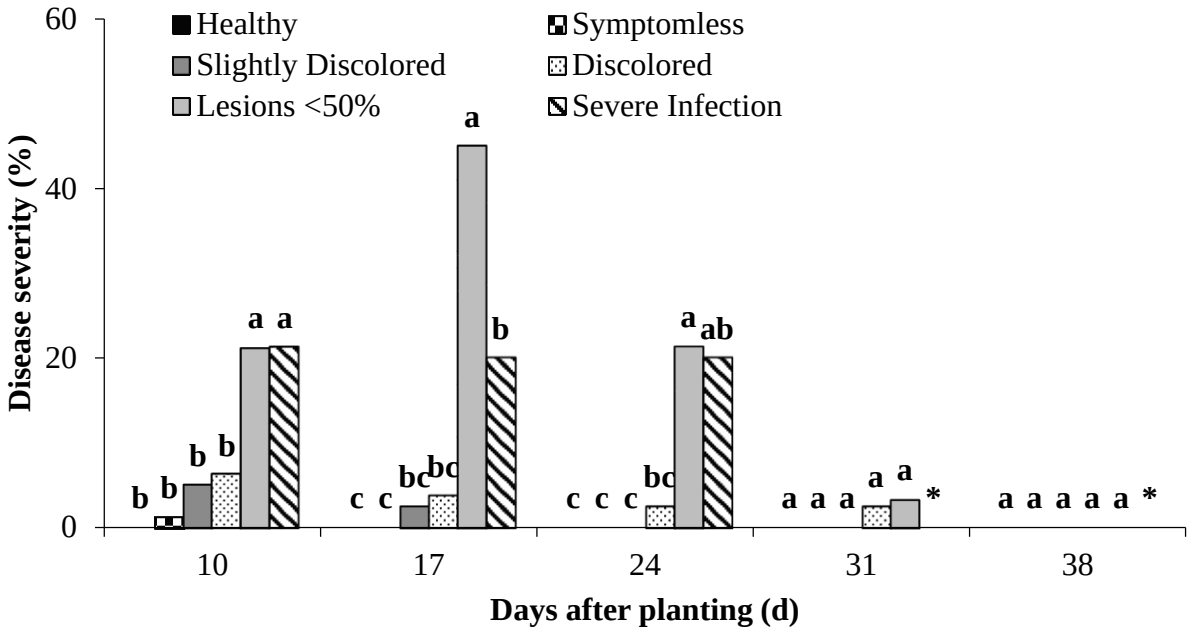

Figure 2.6. Combined analysis of disease severity ratings (%) by week across six asymptomatic and symptomatic seed categories and two greenhouse trials. Columns labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05). *denotes no tissue available

Levene's test for HOV was not significant between trial A and B for root weight (P = 0.5465) and root length (P = 0.1431) and no interaction between trials and seed category was observed for either parameter (P = 0.9829; P = 0.3624); therefore, data from the two trials were combined for further analyses. The general trend was that root weight and length decreased with increasing seed symptom severity, although not significantly (Table 2.5; Table 2.6). Significant differences in root length were observed at 10, 17, and 24 DAP with an overall trend of decreasing root length with increasing seed symptom severity (Table 2.6). Levene's test for HOV determined significant differences between trial A and B in plant height (P = 0.0020) and weight (P <.0001), but no interaction between trial and seed categories was observed for plant height (P = 0.2814) and plant weight (P = 0.1628). The significant difference in variation between trials was due to the plants in trial B being larger than the plants in trial A; therefore, data from the two trials were not combined for further analyses. Again, the general trend was an

inverse relationship between seed symptom severity and plant height and weight. Individual analyses from both trial A and B indicated significant differences in plant height between seed categories at some data collection dates (Table 2.7; Table 2.8) and weight significantly differed 10, 17, and 38 DAP in trial A ($P = 0.0248$, $P = 0.0349$, $P = 0.0415$; Table 2.9) and only 31 DAP in trial B ($P = 0.0156$; Table 2.10).

Table 2.5. Combined analysis of root weights (g) over five sampling dates across six asymptomatic and symptomatic seed categories and two greenhouse trials. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	Lesions <50%	Severe Infection	*P* value
10	2.7 a	2.3 a	3.1 a	2.7 a	2.1 a	0.7 a	0.1021
17	5.2 a	4.4 a	4.0 a	4.3 a	2.2 a	3.4 a	0.1398
24	5.7 a	5.5 a	4.2 a	4.6 a	4.5 a	3.8 a	0.6983
31	4.6 a	5.4 a	4.4 a	4.1 a	2.5 a	*	0.4469
38	3.4 a	5.4 a	3.7 a	3.5 a	6.9 a	*	0.4720

[a] DAP = days after planting
*denotes no tissue available

Table 2.6. Combined analysis of root lengths (cm) over five sampling dates across six asymptomatic and symptomatic seed categories and two greenhouse trials. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	Lesions <50%	Severe Infection	*P* value
10	16.5 a	14.6 a	16.5 a	15.1 a	14.0 a	9.0 b	0.0349
17	23.0 a	21.0 ab	21.1 ab	22.1 ab	11.9 c	16.7 bc	0.0016
24	26.4 ab	28.3 a	21.1 bc	23.3 abc	20.3 bc	19.1 c	0.0324
31	24.4 a	26.4 a	27.3 a	20.6 a	19.1 a	*	0.2197
38	19.3 a	23.3 a	21.8 a	14.7 a	18.5 a	*	0.4044

[a] DAP = days after planting
*denotes no tissue available

Table 2.7. Greenhouse trial A plant heights (cm) across asymptomatic and symptomatic seed categories over five sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	Lesions <50%	Severe Infection	*P* value
10	29.8 a	28.0 a	30.9 a	25.1 a	27.3 a	16.9 a	0.0637
17	34.1 ab	37.4 ab	38.7 a	37.7 ab	18.9 c	29.4 b	0.0003
24	38.0 ab	44.0 a	33.3 ab	32.9 ab	27.0 b	27.0 b	0.0266
31	34.1 a	37.2 a	37.8 a	28.2 a	25.3 a	*	0.0960
38	24.4 a	37.3 a	32.2 a	27.2 a	31.0 a	*	0.1322

[a] DAP = days after planting
*denotes no tissue available

Table 2.8. Greenhouse trial B plant heights (cm) across asymptomatic and symptomatic seed categories over five sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	Lesions <50%	Severe Infection	*P* value
10	25.1 a	22.3 a	21.5 ab	24.8 a	16.8 b	16.8 b	0.0041
17	36.7 a	29.7 a	29.2 a	35.4 a	22.8 a	23.6 a	0.0573
24	45.4 a	42.1 a	47.3 a	47.1 a	35.6 a	33.3 a	0.2828
31	51.7 a	63.8 a	54.8 ab	49.0 a	29.7 b	*	0.0350
38	52.2 ab	77.0 a	55.7 ab	29.4 b	46.0 ab	*	0.0349

[a] DAP = days after planting
*denotes no tissue available

Table 2.9. Greenhouse trial A plant weights (g) across asymptomatic and symptomatic seed categories over five sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	Lesions <50%	Severe Infection	*P* value
10	6.0 a	5.3 a	7.5 a	4.9 a	7.4 a	1.3 b	0.0248
17	9.8 ab	10.8 a	9.0 ab	9.1 ab	4.0 c	6.3 bc	0.0349
24	8.8 a	10.0 a	7.6 a	5.9 a	6.6 a	5.9 a	0.1121
31	4.9 a	5.9 a	5.1 a	3.6 a	1.2 a	*	0.0879
38	1.2 b	6.2 ab	5.1 ab	2.5 ab	7.4 a	*	0.0415

[a] DAP = days after planting
*denotes no tissue available

Table 2.10. Greenhouse trial B plant weights (g) across asymptomatic and symptomatic seed categories over five sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	Lesions <50%	Severe Infection	*P* value
10	3.5 a	2.9 a	2.8 a	4.9 a	2.0 a	1.7 a	0.1321
17	7.7 a	6.0 a	4.9 a	6.8 a	2.7 a	4.6 a	0.3393
24	12.0 a	11.3 a	14.0 a	11.8 a	7.1 a	6.4 a	0.3429
31	18.2 ab	23.0 a	17.4 ab	12.5 bc	8.6 c	*	0.0156
38	16.7 a	24.6 a	15.0 a	10.5 a	21.7 a	*	0.1797

[a] DAP = days after planting
*denotes no tissue available

C. lindemuthianum quantification in stem tissue from greenhouse trials. Using the ClF1527/ClR1609 real-time qPCR assay, *C. lindemuthianum* was detected as early as 10 DAP from stem tissue grown from seeds in the discolored, lesions covering <50% of the seed surface, and severe infection seed categories (Table 2.11; Table 2.12). Real-time qPCR fungal detection data from trial A and trial B could not be combined based on Levene's test for HOV ($P = 0.0004$) and no interaction between trials and seed category was observed ($P = 0.1043$). Significant differences were observed between seed categories in trial A only 10 DAP, but not at any date in trial B. Detection generally increased with increasing seed severity at 10 DAP, but detection after 10 DAP was inconsistent, possibly due to cross-contamination from watering techniques and pest control as well as reductions in sample size. No fungal growth was observed on solid media except from 2 plants collected 10 and 17 DAP in greenhouse trial B. Levene's test for HOV determined significant differences between trial A and B for the level of pathogen DNA detected in the stem tissue ($P = 0.0001$) and no interaction between trials and seed category was observed ($P = 0.1552$), and therefore, these data were not combined (Table 2.13; Table 2.14). As has been demonstrated in other greenhouse data parameters, significant increases in pathogen quantity were observed with increasing seed symptom severity, but only early in the

trial. The detection incidence of C. *lindemuthianum* in stem tissue was positively and significantly correlated to the level of pathogen DNA quantified in seed (r = 0.9946; *P* = 0.0054) and the level of pathogen DNA quantified in stem tissue (r = 0.4168; *P* = 0.0382). The relationship between the level of pathogen colonization in seed and the level of pathogen colonization in stem was positively correlated (r = -0.9431); however, the relationship was not significant (*P* = 0.0569).

Table 2.11. Pathogen detection incidence (%) using real-time qPCR in stem tissue from greenhouse trial A across five data sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	Lesions <50%	Severe Infection	*P* value
10	0.0 b	0.0 b	0.0 b	25.0 b	25.0 b	100.0 A	<.0001
17	25.0 a	50.0 a	12.5 a	25.0 a	62.5 a	75.0 A	0.0915
24	0.0 a	12.5 a	25.0 a	12.5 a	62.5 a	25.0 A	0.1901
31	12.5 a	0.0 a	0.0 a	12.5 a	0.0 a	*	0.4546
38	50.0 a	50.0 a	62.5 a	100.0 a	100.0 a	*	0.6030

[a] DAP = days after planting
*denotes no tissue available

Table 2.12. Pathogen detection incidence (%) using real-time qPCR in stem tissue from greenhouse trial B across five data sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	Lesions <50%	Severe Infection	*P* value
10	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	25.0 a	0.5289
17	12.5 a	12.5 a	33.3 a	12.5 a	0.0 a	50.0 a	0.4210
24	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	.
31	37.5 a	0.0 a	33.3 a	0.0 a	33.3 a	*	0.5260
38	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	*	.

[a] DAP = days after planting
*denotes no tissue available

Table 2.13. Level of pathogen colonization (ng/µl) in stem tissue from greenhouse trial A across five data sampling dates as determined by real-time qPCR. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

	Pathogen colonization (ng/µl)												
DAP[a]	Healthy		Symptomless		Slightly Discolored		Discolored		Lesions <50%		Severe Infection		*P* value
10	0.00	c	0.00	c	0.00	c	0.15	bc	0.19	b	0.53	a	<.0001
17	0.06	c	0.14	bc	0.04	c	0.06	c	0.24	b	0.37	a	<.0001
24	0.00	a	0.04	a	0.17	a	0.12	a	0.18	a	0.06	a	0.1046
31	0.12	a	0.00	a	0.00	a	0.03	a	0.00	a	*		0.2350
38	0.50	c	0.60	c	0.94	bc	1.63	ab	1.88	a	*		0.0137

[a] DAP = days after planting
*denotes no tissue available

Table 2.14. Level of pathogen colonization (ng/µl) in stem tissue from greenhouse trial B across five data sampling dates as determined by real-time qPCR. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

	Pathogen colonization (ng/µl)												
DAP[a]	Healthy		Symptomless		Slightly Discolored		Discolored		Lesions <50%		Severe Infection		*P* value
10	0.00	b	0.00	b	0.00	b	0.00	b	0.00	b	0.05	a	0.0014
17	0.18	a	0.21	a	0.20	a	0.14	a	0.00	a	0.24	a	0.5479
24	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a	.
31	0.05	a	0.00	a	0.07	a	0.00	a	0.14	a	*		0.0509
38	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a	*		.

[a] DAP = days after planting
*denotes no tissue available

Evaluation of 2013 field trial data. Due to poor environmental conditions, emergence was very poor, ranging from 6% to 26% across seed categories 1 through 4. Therefore, only emergence, plant height and weight, root length and weight, and real-time qPCR data were able to be collected at the trial conducted near Sykeston, ND. No disease developed and the trial was destroyed prior to crop maturity. Discolored seed had significantly lower emergence than all other categories and slightly discolored was significantly lower than healthy and symptomless (Fig. 2.7). Healthy seed, which was obtained from a separate seed source, and symptomless seed had the highest level of emergence and were not significantly different from each other. The

general trend was that as seed symptom severity increased, fresh weight and plant height decreased. However, significant differences between seed categories in fresh weight (P = 0.0052; Table 2.15) and plant height were only observed at 28 DAP (P = 0.0010; Table 2.16). Root lengths were only collected at 28 and 35 DAP, and no significant differences between seed categories were observed at either date (Table 2.17). No symptoms of anthracnose were found at any time prior to crop destruction and real-time qPCR did not detect any level of the pathogen in stem samples.

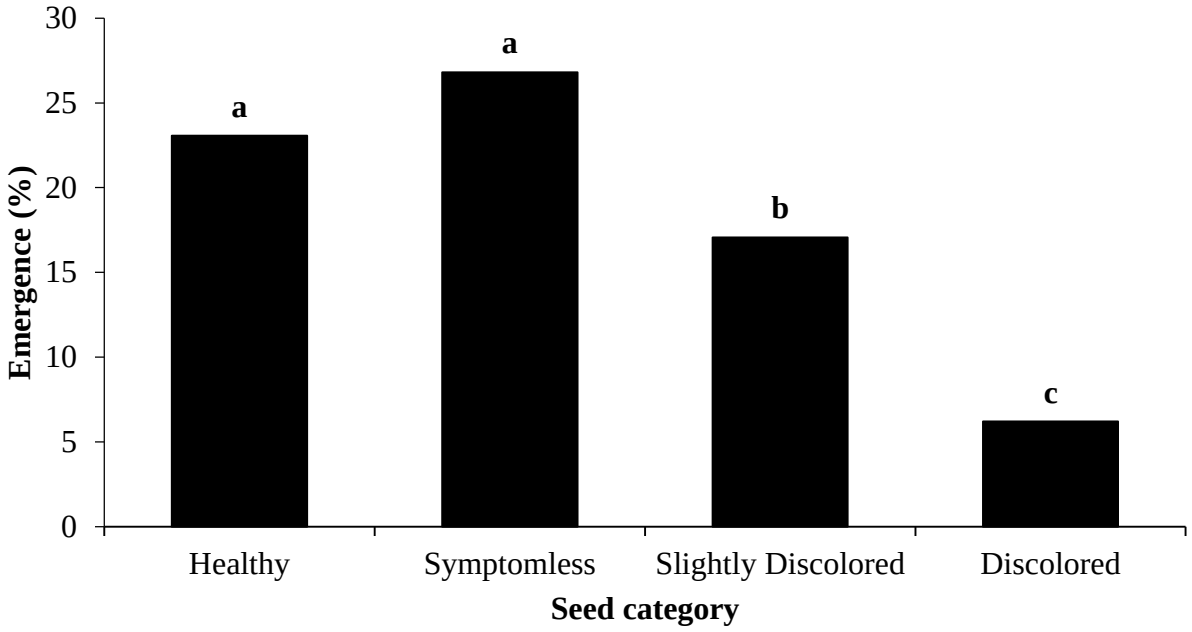

Figure 2.7. Seedling emergence (%) for 2013 field trial across six asymptomatic and symptomatic seed categories. Columns labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

Table 2.15. Fresh weights (g) of plants collected during the 2013 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	*P* value
15	3.7 a	3.5 a	3.3 a	3.0 a	0.2606
21	7.6 a	7.7 a	8.7 a	7.8 a	0.2340
28	21.0 ab	26.1 a	20.5 b	17.0 b	0.0052
35	36.8 a	51.1 a	45.8 a	43.2 a	0.1448

[a] DAP = days after planting

Table 2.16. Plant height (cm) of plants collected during the 2013 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	*P* value
15	7.8 a	8.0 a	7.7 a	7.7 a	0.9673
21	11.4 a	10.3 a	10.9 a	12.2 a	0.1043
28	14.2 a	15.3 a	13.9 a	12.5 b	0.0010
35	15.8 a	18.4 a	16.4 a	16.2 a	0.0728

[a] DAP = days after planting

Table 2.17. Root lengths (cm) of plants collected during the 2013 field trial across four asymptomatic and symptomatic seed categories and two data sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	*P* value
28	3.4 a	3.7 a	3.4 a	3.2 a	0.4892
35	3.5 a	4.3 a	3.8 a	3.9 a	0.1296

[a] DAP = days after planting

Evaluation of 2014 field trial data. In 2014, a field trial was conducted at the Research Station in Morden, Manitoba, Canada. Seedling emergence in discolored seed was significantly lower than all other categories while the symptomless seed category had the highest emergence rate, significantly higher than that of the healthy seed obtained from a separate seed source (*P* <.0001; Fig. 2.8). Using the real-time qPCR assay with primer pair ClF1527/ClR1609, *C. lindemuthianum* was detected as early as 10 DAP in all seed categories. Significant differences were not observed within seed categories on any sampling date (Fig. 2.9). However, the

detection of the pathogen in healthy seed suggests the presence of field inoculum. Pathogen detection incidence increased significantly as the season progressed beginning at 18 DAP. The quantified level of pathogen DNA followed the same trend as detection incidence, but statistical differences were observed within seed categories at 24 (P = 0.0127) and 32 DAP (P <.0001; Fig. 2.10). Plants grown from symptomless seed had significantly higher colonization than all other seed categories, followed by plants grown from discolored seed which were significantly more colonized than plants grown from healthy and slightly discolored seed.

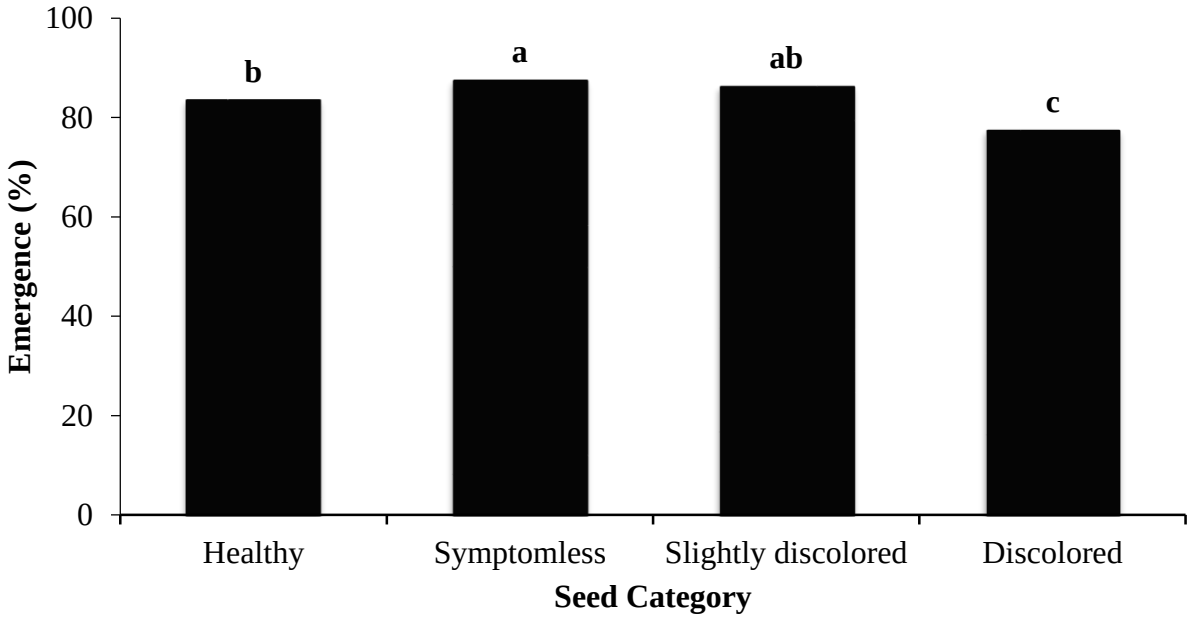

Figure 2.8. Seedling emergence (%) for 2014 field trial across four asymptomatic and symptomatic seed categories. Columns labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

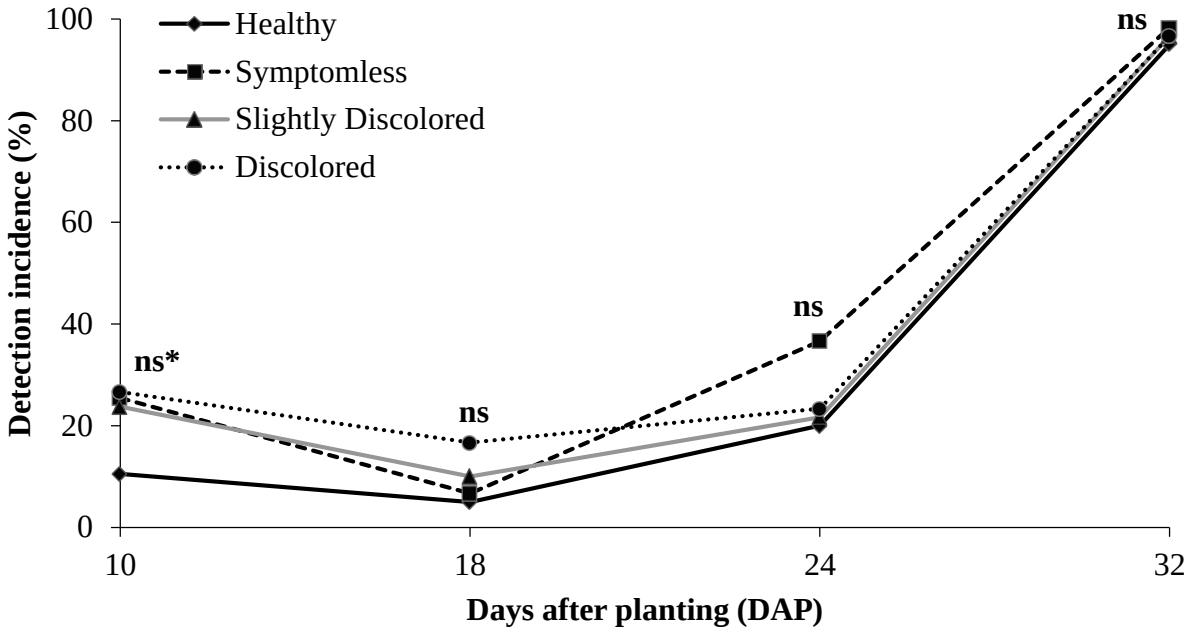

Figure 2.9. Real-time qPCR detection incidence (%) of pathogen DNA in stem tissue collected from the 2014 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates. *ns = not significant

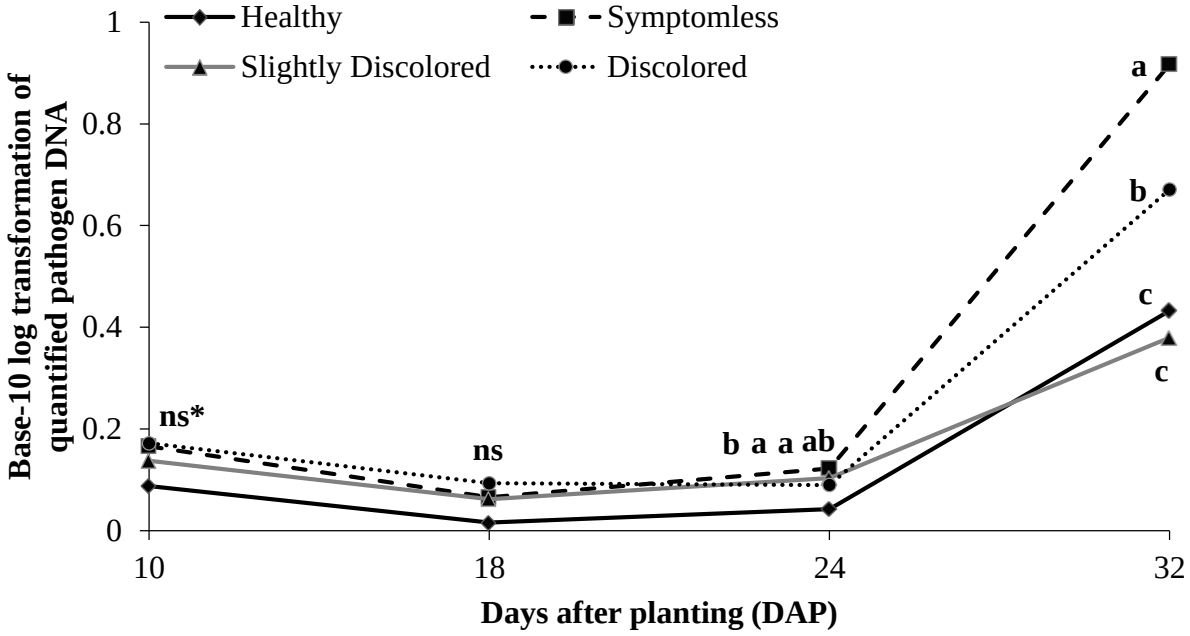

Figure 2.10. Level of pathogen colonization (ng/µl) in 2014 field stem tissue across four asymptomatic and symptomatic seed categories and four data sampling dates as determined by real-time qPCR. Line markers labeled with the same lowercase letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$). *ns = not significant

Plant fresh weight (Table 2.18) and height (Table 2.19) significantly decreased with increasing seed symptom severity at all sample dates but 32 DAP for both parameters. Root length also generally decreased with increasing seed symptom severity, but these differences were significant only at 10 (P = 0.0009) and 24 DAP (P = 0.0144; Table 2.20). Lesions characteristic of anthracnose were found on stems while conducting these plant sample collections; however, plating assays did not recover any fungal growth.

Table 2.18. Fresh weights (g) of plants collected during the 2014 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	*P* value
10	2.7 a	2.5 b	2.1 c	2.0 c	<.0001
18	7.0 a	7.2 a	5.9 b	5.0 c	<.0001
24	15.1 a	14.6 a	11.9 b	12.5 b	0.0014
32	38.2 a	39.1 a	35.6 a	37.9 a	0.6768

[a] DAP = days after planting

Table 2.19. Full heights (cm) of plants collected during the 2014 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	*P* value
10	16.7 a	16.4 a	14.5 B	14.8 b	0.0018
18	20.7 a	20.6 a	19.8 A	18.4 b	0.0016
24	26.6 a	27.0 a	23.8 B	23.2 b	<.0001
32	34.8 a	37.0 a	34.3 A	35.9 a	0.2742

[a] DAP = days after planting

Table 2.20. Root lengths (cm) of plants collected during the 2014 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

DAP[a]	Healthy	Symptomless	Slightly Discolored	Discolored	*P* value
10	8.2 a	7.5 ab	5.8 C	6.4 bc	0.0009
18	9.7 a	9.3 a	8.9 A	8.3 a	0.1008
24	11.8 ab	11.9 a	10.4 bc	10.0 c	0.0144
32	13.2 a	14.1 a	13.3 A	12.8 a	0.4250

[a] DAP = days after planting

After all plant samples were collected, the plots were continuously monitored for anthracnose symptoms. Significant differences between seed categories were observed at 46 (P = 0.0056), 61 (P <0.0001), 75 (P <0.0001) and 82 DAP (P <0.0001; Fig. 2.11). Disease severity at 46 and 61 DAP was less than 7% for all categories but substantial increases in disease severity were observed at 75 and 82 DAP. Across all dates, plants from symptomless seed had the lowest anthracnose severity while plants from discolored seed had the highest, although not always significantly higher than all other categories. Plants from healthy seed remained disease-free until 75 DAP, when less than 1% severity was observed.

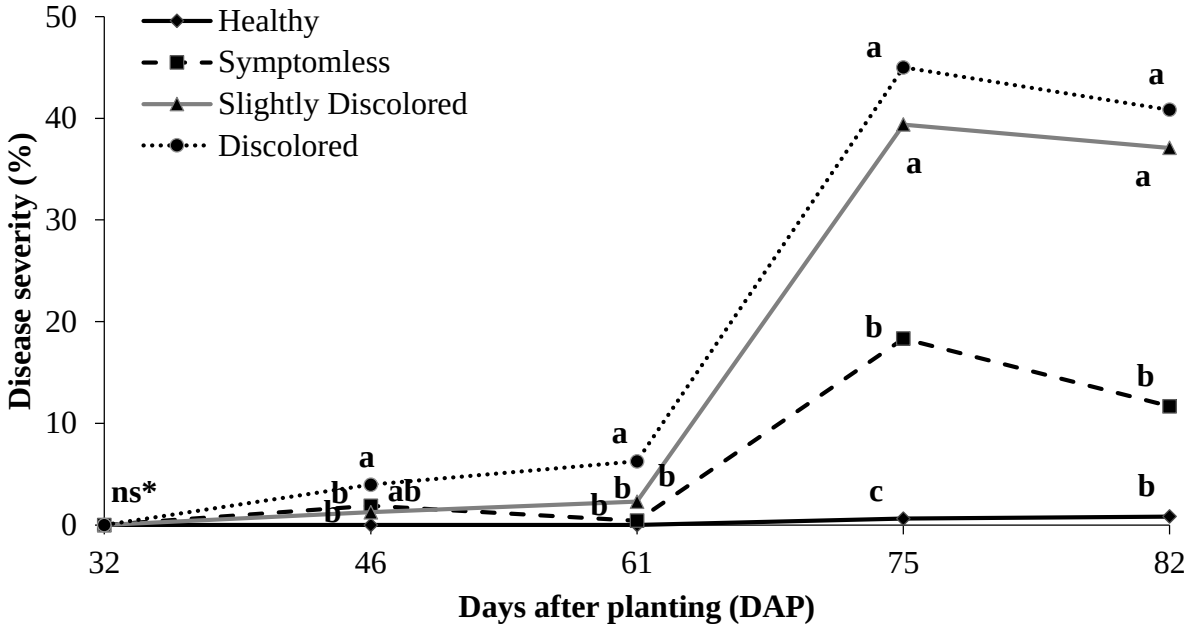

Figure 2.11. Disease severity (%) within plots during the 2014 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates. Line markers labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05). *ns = not significant

Plants were allowed to reach full maturity at which point each plot was harvested. The average seed weight between the seed categories was not significantly different (P = 0.1347; Table 2.21); however, incidence of seed discolor (P <0.0001) and average yield (P = 0.0002) were significantly different among seed categories. Results from a correlation analysis revealed yield and seed discolor were negatively and significantly correlated (r = -0.9807; P = 0.0193).

Table 2.21. Average seed weight of 200 seeds (g), percentage of seeds found to be anthracnose symptomatic out of 200 seeds, and average plot yield (g/plot) after harvest of the 2014 field trial across four asymptomatic and symptomatic seed categories. Rows labeled with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

	Healthy		Symptomless		Slightly Discolored		Discolored		*P* value
Seed weight (g)	78.3	a	77.8	a	77.1	a	74.9	a	0.1347
Symptomatic seed (%)	2.1	b	3.6	b	10.8	a	12.4	a	<.0001
Yield (g)	4971.3	a	5053.0	a	4116.8	b	3761.3	b	0.0002

Comparison of four molecular methods. Evaluations comparing the newly developed assay using ClF1527/ClR1609 and the previously developed assay using ClF432/ClR533 (Chen et al., 2013) on seed flour resulted in differences in detection incidences of the primer pairs. These differences were significant in slightly discolored (P = 0.0097) and discolored seed (P = 0.0471; Fig. 2.12). ClF432/ClR533 detected *C. lindemuthianum* in more seed flour samples from all seed categories than ClF1527/ClR1609, including healthy seed flour used as the negative control. Amplicon sequencing of PCR products from both assays and real-time qPCR amplification curves and melt peaks confirmed that ClF1527/ClR1609 produced the desired product. Evaluation of melt peaks from ClF432/ClR533 indicated two melt peaks were produced instead of the desired single peak (Fig. 2.13). The first peak at approximately 77°C was producing a fluorescent signal leading to false positive results. The same pattern was observed in the negative (water) sample indicating it was positive, whereas the negative sample from

ClF1527/ClR1609 did not amplify. Comparisons of the real-time qPCR assays with and without the respective probes resulted in ClF1527/ClR1609 or ClP1563 consistently reducing the number of false positives in the real-time qPCR compared to assay using ClF432/ClR533 or ClP442 (Table 2.22).

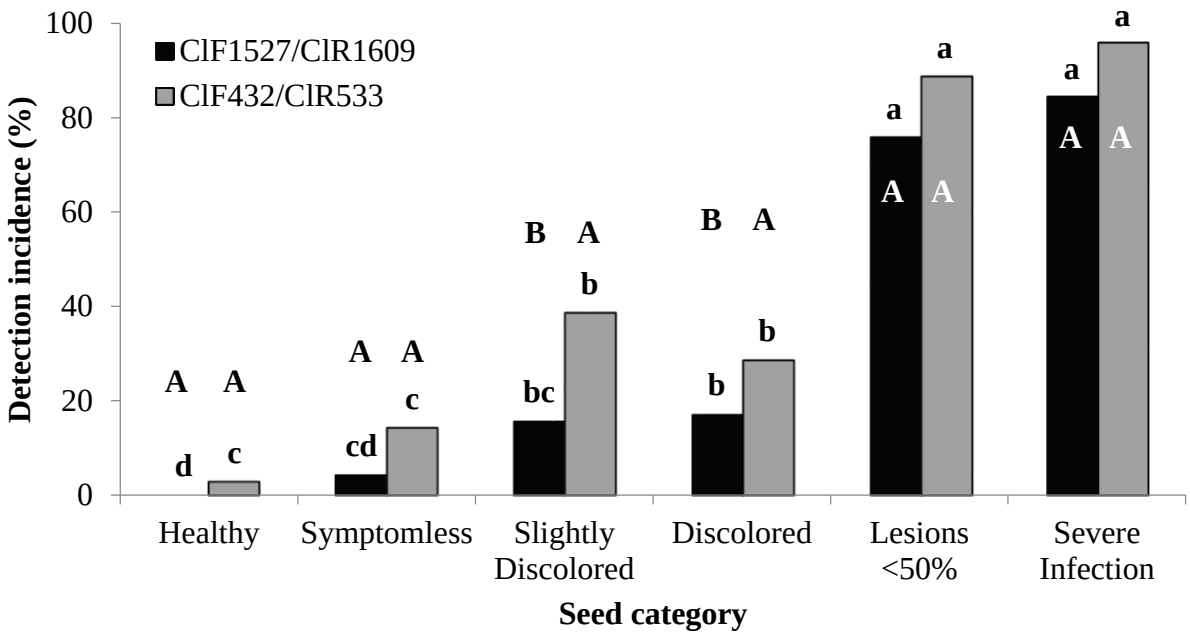

Figure 2.12. Detection (%) of *C. lindemuthianum* in symptomatic and asymptomatic seed using two real-time qPCR assays. Columns labeled with the same lowercase letter compare the seed categories by method and are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$). Columns labeled with the same uppercase letter compare method by seed category are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

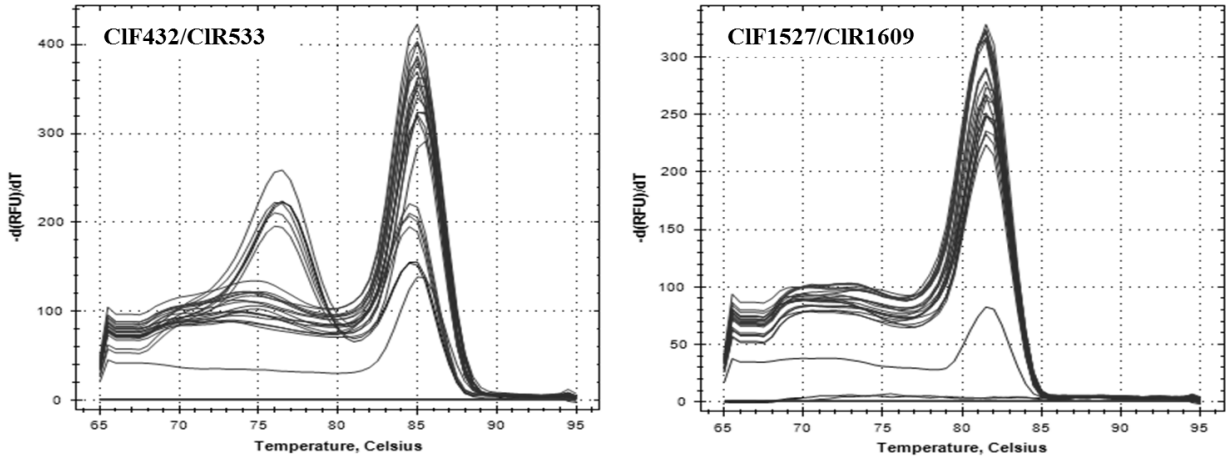

Figure 2.13. Melt peaks for primer pairs ClF432/ClR533 and ClR1527/ClR1609, respectively.

Table 2.22. Relationship between the positive samples of each of the assays represented by a regression coefficient (R^2) and frequencies (%) with which each method detected *Colletotrichum lindemuthianum* DNA within the seed samples.

		Detection frequency (%)			
Real-time qPCR comparisons	R^2	Both +	1+	2+	Both -
ClF432/ClR533 vs ClP442	0.297	36.2	28.2	6.9	28.7
ClR1527/ClR1609 vs ClP442	0.113	23.0	5.2	20.1	51.7
ClP1563 vs ClP442	0.084	18.4	2.9	24.7	54.0
ClF1527/ClR1609 vs ClR432/ClR533	0.233	25.9	2.3	38.5	33.3
ClP1563 vs ClF432/ClR533	0.194	19.5	1.7	44.8	33.9
ClR1527/ClR1609 vs ClP1563	0.943	21.3	6.9	0.0	71.8

Discussion

Anthracnose of dry bean can be extremely devastating, leading to significant yield losses and reduced seed quality. While previous evaluations have determined that disease severity increases with increasing seed symptom severity (Conner et al., 2009; Tu, 1983; Mohammed and Sangchote, 2005, 2007), to our knowledge, no evaluations have been performed to quantify pathogen movement from seed to seedling. The research conducted here supports the previous reports for the relationship between seed symptom severity and anthracnose disease severity in dry bean. However, relationships were also demonstrated between seed symptom severity, disease severity, yield, and other factors, and *C. lindemuthianum* presence and quantity in stem

tissue. This is the first time seed germination and seedling emergence have been demonstrated to be negatively and significantly correlated to the level of pathogen DNA detected in seed as determined by real-time qPCR assays as well as the first time the detection incidence of *C. lindemuthianum* in stem tissue has been correlated to the level of pathogen DNA quantified in seed. Additionally, the pathogen was quantified *in planta* using a novel real-time qPCR assay that reduced the incidence of false positives which would have an impact on seed lot rejections 10 DAP.

In the greenhouse trials, each plant was incubated in a favorable environment with cool temperatures and submitted to a regular watering regime. This may have contributed to early detection of *C. lindemuthianum* in the stem tissue, as early as 10 DAP in some treatments. In the 2013 field trial, environmental conditions were not favorable for pathogen growth and disease development throughout the growing season. From June 1, 12 days prior to planting, to July 17, when stem sampling was completed, a total of 4.1 cm of rain fell at the research station in Carrington, approximately ten miles from the trial site. Total precipitation in one event was never greater than 1.6 cm, the average over the 11 rain events was 0.37 cm and half of these rain events occurred before planting. The maximum temperature ranged from 13°C to as high as 31°C. As expected, no anthracnose developed in these plots, and no pathogen was detected in stem tissue from any seed symptom level, even early in the growing season. In 2014, prior to 10 DAP (June 2), the weather was favorable for disease development. Between 10 and 18 DAP, temperatures remained cool with daily high temperatures ranging from 17°C to 25°C, however, a dry period was observed. The weather remained cool and dry until mid-July, approximately 61 DAP, when rains became frequent, driving heavy dew that sometimes left the canopy wet for 10 or 12 hours in a 24 hour period. This favorable weather resulted in increasing disease severity.

However, the detection, or lack thereof, of *C. lindemuthianum* in stem tissue has not been demonstrated to be influenced by environmental conditions. This was particularly notable early in the growing season when stem samples were taken in the greenhouse and field trials, about the time the first true leaves are emerging.

C. lindemuthianum is a seed-borne fungus that penetrates plant tissue including the seed coat (Singh and Mathur, 2004). Therefore, seed infection is common and plays a major role as the main source of inoculum for disease outbreaks. Previous research has demonstrated the significance of the location of the pathogen in the seed. In most instances (74.4%), infection was limited to the seed coat (Mohammed and Sangchote, 2005). Less commonly, the cotyledon (21.3%), and embryo axis (4.3%) also contained *C. lindemuthianum*, and this was only observed in areas that suffered severe anthracnose outbreaks (Mohammed and Sangchote, 2005). Under cool, moist conditions this pathogen can germinate and infect plant tissue within 2 to 4 days. Epigeal germination brings the source of inoculum above the soil surface, leading to the infection of the hypocotyl if weather conditions are favorable, theoretically allowing for the detection of the pathogen as early as 4 days after emergence via real-time qPCR. Our results indicate that *C. lindemuthianum* was present in stem tissue as early as 10 DAP; however, this was the earliest time point that was evaluated.

As demonstrated by the field trial, as the severity of the seed infection increased, daughter seed infection increased and yield decreased significantly. These results support previous research demonstrating that significance of using even a small proportion of diseased seed on not only yield loss, but loss in crop quality (Conner et al., 2009). Blemishes can lead to a seed lot being rejected for canning, processing, or for use as seed for the next growing season. In the seed lots evaluated as part of this study, approximately 5% of symptomless seed and 17%

of slightly discolored and discolored seed was infected with *C. lindemuthianum*, depending on method of detection. Given that no significant reductions in emergence and little differences in general plant development were observed, it is safe to conclude that functional plants are generated from these infected seeds. The results of this situation are demonstrated in the disease severity data from the 2014 field trial. Weather conditions were conducive for disease development, pathogen inoculum was present, and more disease developed in those seed categories with more initial pathogen inoculum. This does not explain the lack of significant differences in *C. lindemuthianum* detection incidence or quantity; however, these measurements were completed prior to the presence of visual disease symptoms and quantity detected was low. Future studies should take this into consideration and extend pathogen quantification further into the growing season.

Real-time qPCR has been used for the diagnosis and quantification of several plant pathogens. Pathogen detection in symptomless seed is vital to aid growers when purchasing seed to prevent anthracnose epidemics. Additionally, traditional detection methods are labor intensive and time consuming, while PCR based assays are relatively easy to complete and provide rapid results. Traditional methods like plating tissue on solid growth media or the ISTA protocol can take weeks to complete and return results. A new real-time qPCR assay using primer pairs ClF1527/ClR1609 has proven to be an effective means of detecting *C. lindemuthianum* in dry bean seed and stem tissue based on sensitivity, specificity, and efficiency. This primer pair is located on the single copy nitrate reductase gene that is specific to *C. lindemuthianum*. This primer pair was more accurate when positively identifying pathogen DNA within dry bean seed or tissue samples and appears to be more specific than the previously developed primer pair and traditional methods of detection. Evaluations performed using this real-time qPCR assay led us

to believe false positives may have been amplified using the primer pairs ClF432/ClR533 alone and may not have been as specific as indicated. Based on the real-time qPCR assay comparisons and correlations of the Cq values of the four molecular methods, ClF432/ClR533 had a higher frequency of detecting false positives than ClF1527/ClR1609. Primer pair ClF1527/ClR1609 consistently reduced the number of false positives with or without its associated probe ClP1563. However, the assay developed in this research was only validated with single seed samples.

The new assay using primer pair ClF1527/ClR1609 was validated *in planta* using stem samples from the greenhouse and field trials. The pathogen was detected as early as 10 DAP in both the greenhouse and field assays continuing until the end of the sampling period, indicating the pathogen was present within stem tissue, regardless of symptom expression. Data collected demonstrated that as the seed symptom severity increased, seedling emergence, seed germination, and yield decreased, whereas disease severity, pathogen DNA quantity, and detection incidence generally increased.

In previous studies, it has been demonstrated that roots are not affected by the pathogen. Data collected in this study exhibited an overall trend of decreasing root length with increasing seed symptom severity; however, the root weight was not affected by the increasing seed symptom severity although pathogen quantification was not attempted from root tissue. Foliar height was affected by the pathogen leading to generally shorter plants in the seed categories with higher infection. This could be due to the poor establishment affecting the overall growth of the plant.

In conclusion, this study demonstrates the importance of using clean seed whenever planting dry beans. It was shown that the level of *C. lindemuthianum* DNA in seed directly impacted seed germination and seedling emergence and viable plants can be grown from infected

seed. Inoculum present above the soil surface or on the cotyledons of emerged plants grown from infected seed may start an epidemic. Real-time qPCR provides faster results than traditional methods of pathogen detection. This study demonstrated that the detection incidence and quantity of *C. lindemuthianum* in stem tissue was related to the level of pathogen DNA quantified in seed.

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APPENDIX A. VIRULENCE PHENOTYPES OF NORTH DAKOTA *COLLETOTRICHUM LINDEMUTHIANUM* ISOLATES ON A STANDARD SET OF DRY BEAN DIFFERENTIALS

Table A.1. Virulence phenotypes of *C. lindemuthianum* isolates collected in North Dakota on a standard set of dry bean differentials.

Year	County	Field	Isolate	Differential												Race
				Mich[a]	MDRK[b]	PM[c]	Cornell[d]	Widusa	Kaboon	Mex[e]	PI[f]	TO	TU	AB[g]	G 2333	
2012	Wells	1	2	6-4[h]	0	0	9/5-7	0	0	4-6	0	0	0	0	0	73
2012	Wells	1	5	4	0	0	4-8	0	0	0	0	0	0	0	0	9
2012	Wells	1	7	4-6	0	0	9/5	0/5	0	4-7	0	0	0	0	0	73
2012	Wells	2	2	4/3-5	0	0	4/9	0	0	1-4	0	0	0	0	0	9
2012	Wells	2	4	2	0	0	4/7-9	0	0	4/1	0	0	0	0	0	72
2012	Wells	2	8	4/3-6	0	0	3/6-9	0	0	4	0	0	0	0	0	73
2012	Wells	3	3	4-5/2	0	0	9	0	0/4	7/4-8	0	0	0	0	0	73
2012	Wells	3	8	5-3	0	0	7/9	0	0/4	4-7	0	0	0	0	0	73
2012	Wells	3	15	5/1-3	0	0	5-9	0	0/4	4-7	0	0	0	0	0	73
2012	Wells	4	1	4-5/7	0	0	6/9	0	0	5/4-6	0	0	0	0	0	73
2012	Wells	4	2	4	0	0	5-9	0	0	4	0	0	0	0	0	73
2012	Wells	5	2	4	0	0	7	0	0	4	0	0	0	0	0	73
2012	Wells	5	15	4-5	0	0	5-9	0	0	4-6	0	0	0	0	0	73
2012	Wells	5	22	4/9	0	0	4-5/9	0	0	4/6	0	0	0	0	0	73
2012	Wells	6	4	4-9	0	0	9-5	0	0	2-1	0	0	0	0	0	9
2012	Wells	6	7	4/6	0	0	9/7	0	0	4	0	0	0	0	0	73
2012	Wells	6	9	4	0	0	9/5-7	0	0	4-6	0	0	0	0	0	73

[a] Michelite, [b] Michigan Dark Red Kidney, [c] Perry Marrow, [d] Cornell 49242, [e] Mexico 222, [f] PI 207262, [g] AB 136

[h] Reactions observed on seedlings at 10 d post-inoculation using a 0 to 9 scale according to Balardin et al. (1997), where scores of 0, 1, 2, or 3 are considered resistant and scores of 4, 5, 6, 7, 8, or 9 are considered susceptible. "-" indicates scores within a range. "/" indicates scores were heterogeneous with dominant type given first.

Table A.1. Virulence phenotypes of *C. lindemuthianum* isolates collected in North Dakota on a standard set of dry bean differentials (continued).

Year	County	Field	Isolate	Differential												Race
				Mich[a]	MDRK[b]	PM[c]	Cornell[d]	Widusa	Kaboon	Mex[e]	PI[f]	TO	TU	AB[g]	G 2333	
2012	Wells	7	4	4-5/9	0	0	5/7-9	0	0	4-7	0	0	0	0	0	73
2012	Wells	7	5	2-4/9	0	0	9/4-5	0	0	4-6	0	0	0	0	0	73
2012	Wells	7	8	4-5	0	0	9/7	0	0	4-9	0	0	0	0	0	73
2012	Wells	8	3	4/2	0	0	9/2	0	0	4-1	0	0	0	0	0	73
2012	Wells	8	4	4	0	0	8/4-5	0	0	5-4	0	0	0	0	0	73
2012	Wells	8	5	1	0	0	4/9	0	0	3/5-7	0	0	0	0	0	72
2012	Wells	9	1	4-3	0	0	9/4-6	0	0	4	0	0	0	0	0	73
2012	Wells	9	2	4-6	0	0	5-6/9	0	0	4-5	0	0	0	0	0	73
2012	Foster	10	A	4-3/9	0	0	9-4	0	0	4-6	0	0	0	0	0	73
2012	Foster	10	B	4	0	0	9-4	0	0	5-3	0	0	0	0	0	73
2012	Foster	10	C	4-5	0	0	9/4-7	0	0	4-6	0	0	0	0	0	73
2012	Foster	10	D	4	0	0	9	0	0	5-6	0	0	0	0	0	73
2012	Foster	10	F	1-0	0	0	5-7	0	0/5	3-6	0	0	0	0	0	72
2012	Foster	10	G	4-5/9	0	0	9/7	0	0/1	4/3	0	0	0	0	0	73
2012	Foster	10	J	4-5/9	0	0	9/5-7	0	0/1	4-7	0	0	0	0	0	73
2012	Foster	10	K	4/5	0	0	5/9	0	0	6/4	0	0	0	0	0	73

[a] Michelite, [b] Michigan Dark Red Kidney, [c] Perry Marrow, [d] Cornell 49242, [e] Mexico 222, [f] PI 207262, [g] AB 136

[h] Reactions observed on seedlings at 10 d post-inoculation using a 0 to 9 scale according to Balardin et al. (1997), where scores of 0, 1, 2, or 3 are considered resistant and scores of 4, 5, 6, 7, 8, or 9 are considered susceptible. "-" indicates scores within a range. "/" indicates scores were heterogeneous with dominant type given first.

APPENDIX B. SUMMARY OF STATISTICAL ANALYSES FOR DETECTION OF *COLLETOTRICHUM LINDEMUTHIANUM* IN DRY EDIBLE BEAN SEED FLOUR

Table B.1. Combined analysis of variance of the detection incidences of *C. lindemuthianum* in dry edible bean seed flour as determined by two real-time qPCR assays across six seed categories and two trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	9.52	0.04	0.8508
Rep (Trial)	6	1593.65	5.94	<.0001
Primer	1	5952.38	22.19	<.0001
Seed category	5	40083.81	149.42	<.0001
Primer x Seed category	5	289.52	1.08	0.3742

Table B.2. Combined analysis of variance of the detection incidences of *C. lindemuthianum* in dry edible bean seed flour as determined by two methods (real-time qPCR assays utilizing primer pair ClF1527/ClR1609 and the International Seed Testing Association protocol 7-006) across six seed categories and two trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	76.19	0.334	0.5617
Rep (Trial)	6	884.55	3.94	0.0016
Method	1	104.76	0.47	0.4963
Seed category	5	16410.55	73.14	<.0001
Method x Seed category	5	162.08	0.72	0.6085

Table B.3. Combined analysis of variance of the detection incidences of *C. lindemuthianum* in dry edible bean seed flour as determined by real-time qPCR assays utilizing forward and reverse primer pairs ClF1527/ClR1609 across two trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	76.19	0.32	0.5707
Rep (Trial)	6	885.71	3.77	0.0027
Seed category	5	19371.43	82.56	<.0001
Trial x Seed category	5	556.19	2.37	0.0486

Table B.4. Combined analysis of variance of the detection incidences of *C. lindemuthianum* in dry edible bean seed flour as determined by real-time qPCR assays utilizing forward and reverse primer pairs ClF432/ClR533 across two trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	171.43	0.65	0.4224
Rep (Trial)	6	915.87	3.48	0.0047
Seed category	5	21001.91	79.84	<.0001
Trial x Seed category	5	571.43	2.17	0.0677

Table B.5. Analysis of variance of the detection incidences of *C. lindemuthianum* in dry edible bean seed flour as determined by International Seed Testing Association protocol 7-006.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	2	20.67	0.67	0.5328
Seed category	5	5910.93	191.91	<.0001

Table B.6. Combined analysis of variance of the levels of pathogen colonization in dry edible bean seed flour as determined by real-time qPCR assays using primer pair ClF1527/ClR1609 across six asymptomatic and symptomatic seed categories and two trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	3.59	9.24	0.0025
Rep (Trial)	6	2.36	6.07	<.0001
Seed category	5	93.23	239.67	<.0001
Trial x Seed category	5	4.82	12.40	<.0001

APPENDIX C. SUMMARY OF STATISTICAL ANALYSES FOR SEED TO SEEDLING ANTHRACNOSE TRANSMISSION GREENHOUSE TRIALS

Table C.1. Combined analysis of variance of seed germination across six levels of asymptomatic and symptomatic seed categories and two greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	760.50	3.23	0.0776
Rep (Trial)	9	764.72	3.24	0.0029
Seed category	5	5138.17	21.8	<.0001
Trial x Seed category	5	338.00	1.43	0.2255

Table C.2. Combined analysis of variance of seedling emergence across six levels of asymptomatic and symptomatic seed categories and two greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	4800.00	14.60	0.0006
Rep (Trial)	3	483.33	1.47	0.2406
Seed category	5	3315.00	10.08	<.0001
Trial x Seed category	5	365.00	1.11	0.3739

Table C.3. Combined analysis of variance of dry bean anthracnose disease severity ratings across six asymptomatic and symptomatic seed categories and two greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	113.84	0.58	0.4462
Rep (Trial)	3	1062.79	5.45	0.0014
Days after planting (DAP)	4	830.77	4.26	0.0027
Seed category	5	1586.91	8.13	<.0001
Trial x Seed category	5	322.62	1.65	0.1493
Trial x DAP	4	260.05	1.33	0.2604
DAP x Seed category	18*	313.43	1.61	0.0576
Trial x DAP x Seed category	18*	72.72	0.37	0.9909

* missing 2 data points

Table C.4. Combined analysis of variance of dry bean root weights across six asymptomatic and symptomatic seed categories and two greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	59.49	8.48	0.0039
Rep (Trial)	3	72.66	10.36	<.0001
Days after planting (DAP)	4	61.42	8.75	<.0001
Seed category	5	16.86	2.40	0.0376
Trial x Seed category	5	0.98	0.14	0.9829
Trial x DAP	4	113.98	16.25	<.0001
DAP x Seed category	18*	7.61	1.09	0.3676
Trial x DAP x Seed category	18*	6.99	1.00	0.4639

* missing 2 data points

Table C.5. Combined analysis of variance of dry bean root lengths across six asymptomatic and symptomatic seed categories and two greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	1	611.24	13.87	0.0002
Rep (Trial)	3	214.02	4.86	0.0027
Days after planting (DAP)	4	913.81	20.73	<.0001
Seed category	5	309.97	7.03	<.0001
Trial x Seed category	5	48.37	1.10	0.3624
Trial x DAP	4	440.81	10.00	<.0001
DAP x Seed category	18*	66.79	1.52	0.0850
Trial x DAP x Seed category	18*	43.65	0.99	0.4716

* missing 2 data points

Table C.6. Analysis of variance of dry bean fresh weights across six asymptomatic and symptomatic seed categories from trial A.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	219.29	16.77	<.0001
Days after planting (DAP)	4	107.79	8.24	<.0001
Seed category	5	46.32	3.54	0.0048
DAP x Seed category	18*	21.96	1.68	0.0494

* missing 2 data points

Table C.7. Analysis of variance of dry bean plant heights across six asymptomatic and symptomatic seed categories from trial A.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	637.49	8.91	<.0001
Days after planting (DAP)	4	317.08	4.43	0.0021
Seed category	5	470.04	6.57	<.0001
DAP x Seed category	18*	112.19	1.57	0.0761

* missing 2 data points

Table C.8. Analysis of variance of dry bean fresh weights across six asymptomatic and symptomatic seed categories from trial B.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	53.67	2.52	0.0621
Days after planting (DAP)	4	777.87	36.54	<.0001
Seed category	5	90.83	4.27	0.0015
DAP x Seed category	18*	44.06	2.07	0.0122

* missing 2 data points

Table C.9. Analysis of variance of dry bean plant heights across six asymptomatic and symptomatic seed categories from trial B.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	134.51	1.39	0.2488
Days after planting (DAP)	4	3627.55	37.62	<.0001
Seed category	5	678.82	7.04	<.0001
DAP x Seed category	18*	282.51	2.93	0.0003

* missing 2 data points

Table C.10. Analysis of variance of the detection incidences of *C. lindemuthianum* in dry edible bean stem tissue as determined by real-time qPCR assays utilizing forward and reverse primer pairs ClF1527/ClR1609 across six asymptomatic and symptomatic seed categories for trial A.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	2729.41	2.72	0.0506
Days after planting (DAP)	4	10557.89	10.51	<.0001
Seed category	5	6304.11	6.28	<.0001
DAP x Seed category	18*	1535.37	1.53	0.1042

* missing 2 data points

Table C.11. Analysis of variance of the detection incidences of *C. lindemuthianum* in dry edible bean stem tissue as determined by real-time qPCR assays utilizing forward and reverse primer pairs ClF1527/ClR1609 across six asymptomatic and symptomatic seed categories for trial B.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	1200.06	1.73	0.1686
Days after planting (DAP)	4	2244.07	3.24	0.0172
Seed category	5	1026.69	1.48	0.2072
DAP x Seed category	18*	412.26	0.60	0.8907

* missing 2 data points

Table C.12. Analysis of variance of *C. lindemuthianum* colonization in dry edible bean stem tissue as determined by real-time qPCR assays utilizing forward and reverse primer pairs ClF1527/ClR1609 across six asymptomatic and symptomatic seed categories for trial A.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.23	2.13	0.0958
Days after planting (DAP)	4	6.40	58.74	<.0001
Seed category	5	1.14	10.45	<.0001
DAP x Seed category	18*	0.45	4.17	<.0001

* missing 2 data points

Table C.13. Analysis of variance of *C. lindemuthianum* colonization in dry edible bean stem tissue as determined by real-time qPCR assays utilizing forward and reverse primer pairs ClF1527/ClR1609 across six asymptomatic and symptomatic seed categories for trial B.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.08	2.81	0.0403
Days after planting (DAP)	4	0.24	8.63	<.0001
Seed category	5	0.01	0.49	0.7813
DAP x Seed category	18*	0.02	0.77	0.7389

* missing 2 data points

APPENDIX D. SUMMARY OF STATISTICAL ANALYSES FOR THE 2013 SEED TO SEEDLING ANTHRACNOSE TRANSMISSION FIELD TRIAL

Table D.1. Analysis of variance of seedling emergence across four levels of asymptomatic and symptomatic seed categories in the 2013 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	235.84	7.87	<.0001
Seed category	3	972.98	32.49	<.0001

Table D.2. Analysis of variance of dry bean fresh weights collected during the 2013 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	3083.91	8.11	<.0001
Days after planting (DAP)	3	68314.15	179.54	<.0001
Seed category	3	1200.15	3.15	0.0243
DAP x Seed category	9	498.94	1.31	0.2269

Table D.3. Analysis of variance of dry bean plant heights collected during the 2013 field trial across four asymptomatic and symptomatic seed categories and four data sampling dates.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	153.18	10.07	<.0001
Days after planting (DAP)	3	2841.50	186.74	<.0001
Seed category	3	46.87	3.08	0.0269
DAP x Seed category	9	30.05	1.97	0.0395

Table D.4. Analysis of variance of dry bean root lengths collected 28 and 35 days after planting during the 2013 field trial across four asymptomatic and symptomatic seed categories.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	9.60	3.20	0.0076
Days after planting (DAP)	3	22.36	7.45	0.0066
Seed category	3	6.42	2.14	0.0950
DAP x Seed category	9	2.41	0.80	0.4934

APPENDIX E. SUMMARY OF STATISTICAL ANALYSES FOR THE 2014 SEED TO SEEDLING ANTHRACNOSE TRANSMISSION FIELD TRIAL

Table E.1. Analysis of variance of seedling emergence across four levels of asymptomatic and symptomatic seed categories in the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	7.08	0.46	0.8061
Seed category	3	240.25	15.49	<.0001

Table E.2. Analysis of variance of the detection incidences of *C. lindemuthianum* in dry edible bean stem tissue across four levels of asymptomatic and symptomatic seed categories and four data sampling dates as determined by real-time qPCR assays utilizing forward and reverse primer pairs ClF1527/ClR1609 in the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	1569.17	5.55	0.0002
Days after planting (DAP)	3	37313.89	132.08	<.0001
Seed category	3	1308.33	1.54	0.2102
DAP x Seed category	9	1250.00	0.49	0.8758

Table E.3. Analysis of variance of *C. lindemuthianum* colonization in dry edible bean stem tissue across four levels of asymptomatic and symptomatic seed categories and four data sampling dates as determined by real-time qPCR assays utilizing forward and reverse primer pairs ClF1527/ClR1609 in the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	6.21	47.67	<.0001
Days after planting (DAP)	3	30.99	237.7	<.0001
Seed category	3	2.86	21.91	<.0001
DAP x Seed category	9	1.57	12.02	<.0001

Table E.4. Analysis of variance of dry bean fresh weights collected across four levels of asymptomatic and symptomatic seed categories and four data sampling dates during the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	19.26	0.26	0.9324
Days after planting (DAP)	3	60421.27	830.11	<.0001
Seed category	3	241.82	3.32	0.0193
DAP x Seed category	9	36.39	0.50	0.8751

Table E.5. Analysis of variance of dry bean plant heights collected across four levels of asymptomatic and symptomatic seed categories during the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	312.69	9.30	<.0001
Days after planting (DAP)	3	17717.38	526.88	<.0001
Seed category	3	291.83	8.68	<.0001
DAP x Seed category	9	50.65	1.51	0.1409

Table E.6. Analysis of variance of dry bean root lengths collected across four levels of asymptomatic and symptomatic seed categories during the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	7.07	0.47	0.7992
Days after planting (DAP)	3	1772.18	117.69	<.0001
Seed category	3	121.51	8.07	<.0001
DAP x Seed category	9	14.30	0.95	0.4807

Table E.7. Analysis of variance of dry bean anthracnose disease severity rating 46 days after planting across four levels of asymptomatic and symptomatic seed categories during the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	16.04	1.10	0.3678
Seed category	3	65.63	4.49	0.0056

Table E.8. Analysis of variance of dry bean anthracnose disease severity rating 61 days after planting across four levels of asymptomatic and symptomatic seed categories during the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	7.76	0.48	0.7933
Seed category	3	195.40	11.98	<.0001

Table E.9. Analysis of variance of dry bean anthracnose disease severity rating 75 days after planting across four levels of asymptomatic and symptomatic seed categories during the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	1327.29	3.30	0.0090
Seed category	3	9939.58	24.69	<.0001

Table E.10. Analysis of variance of dry bean anthracnose disease severity rating 82 days after planting across four levels of asymptomatic and symptomatic seed categories during the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	1761.67	4.87	0.0006
Seed category	3	9084.38	25.10	<.0001

Table E.11. Analysis of variance of dry bean seed weight of 200 seeds across four levels of asymptomatic and symptomatic seed categories after harvest of the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	4.46	0.70	0.6289
Seed category	3	13.70	2.17	0.1347

Table E.12. Analysis of variance of the percentage of dry bean seeds found to be anthracnose symptomatic out of 200 seeds across four levels of asymptomatic and symptomatic seed categories after harvest of the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	27.67	1.45	0.2627
Seed category	3	632.61	33.22	<.0001

Table E.13. Analysis of variance of dry bean plot yield across four levels of anthracnose asymptomatic and symptomatic seed categories after harvest of the 2014 field trial.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	5	61002.68	0.33	0.8849
Seed category	3	2436065.38	13.31	0.0002