EVALUATION OF FUNGICIDE SENSITIVITY AND FORMS OF RESISTANCE OF SELECTED *DIAPORTHE* SPECIES IN SOYBEAN (*GLYCINE MAX* L.)

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

In U.S, *Diaporthe* species caused yield losses of 0.2 million metric tons in soybean in 2022. Quinone outside inhibitor fungicides carry high risk of fungicide resistance and may use for managing *Diaporthe*. In this study, isolates of *D.aspalathi, D.caulivora* and *D.longicolla* from 16 U.S. states were tested for their sensitivity to azoxystrobin. Significant effect of isolates (P<0.05) was observed on effective concentration at which mycelial growth was inhibited by 50% (EC_{50}). The results showed the presence of sensitive *Diaporthe* isolates in soybean to azoxystrobin. Moreover, resistance to stem and seed infection by *D.longicolla* was evaluated in 39 soybean accessions. Significant effect of genotypes was observed on disease severity of stem and seed infection (P<0.05). Correlation between disease severity of stem and seed infection was non-significant. Results indicate defense mechanism against *D.longicolla* during stem and seed infection may differ. These findings indicate need to determine alternative fungicide chemistries and develop *Diaporthe*-resistant soybean varieties.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: LITERATURE REVIEW

Soybean

Origin and history

Soybean [*Glycine max* (L.) Merr.] is a legume crop, which has its origin dated back in 1700 to 1100 B.C. in Northeastern China (Hartman et al. 2011) as an important crop. The crop was domesticated from the wild species, *Glycine soja* Sieb. & Zucc., which had vine growth habits and produced black, small seeds (Anderson et al. 2019). By the 16th century A.D., soybeans had spread to other Asian countries (Hartman et al. 2011). Soybean was introduced to Europe for ornamental purposes, during 1739 in France and in 1790 in England (Hartman et al. 2011). Although the first documented report of soybean growing in the United States was by Samuel Bowen in Georgia in 1765 (Hymowitz & Hartan 1983), it took another century for the increase in the acreage of soybean in the country [Hartman et al. 2011, Anderson et al. 2019, United States Department of Agriculture-National Agricultural Statistics Service (USDA-NASS 2023)]. Today, the U.S. is the second largest soybean producer in the world (122 million metric tons, 30% of the world's production), after Brazil (163 million MT, 40% of the world's production) according to USDA- Foreign Agricultural Service (FAS) (2023).

The substantial protein (40 to 41% of the seed dry weight) and fat content (8 to 24% of the seed dry weight) of soybean has led to a surge in demand during the past century (Medic et al. 2014). In the U.S. soybean is grown in the Eastern half, from the Gulf of Mexico to midwestern U.S. states, where the crop is grown as a full-season, spring-seeded crop (Wilcox 2004). Within the United States, Iowa ranks first in production (14%), followed by Illinois (13%) and Minnesota (9%) (USDA-FAS 2023). However, in the U.S., areas south to 35 ° N latitude, the crop is

cultivated as second crop followed by rice (*Oryza sativa* L), wheat (*Triticum aestivum* L.) or winter canola (*Brassica napus L*.) in late June or early July (Wilcox 2004).

Plant growth stages and development

Soybean plant growth is differentiated into two phases; the vegetative one (V phase), which includes the period from emergence to flowering, and the reproductive phase (R phase) beginning from flowering to pod maturation. The growth identification is essentially based on the node, leaf, flower, and pod development. A node is a part of the stem where the leaf is attached. A leaf is said to be fully developed when the leaf at the node directly above it (the subsequent younger leaf) has expanded to the point where the two lateral edges on each of the leaflets have largely unrolled and are no longer touching. Soybean growth stages begin from the unifoliate node. The remaining leaves produced are trifoliate, which occurs in an alternating pattern on the stem (Fehr et al. 1971, Kandel and Endres 2023).

Soybean growth stages

1. VE (Emergence) -This growth stage is characterized by the emergence of the seedling from the soil. Having an epigeal germination, cotyledons are pushed through the soil forming an arch called ‘hypocotyl arch’ (Figure 1.1).
2. VC (Cotyledon) - The first true leaves are produced at this stage. The unifoliate leaves are fully unrolled (Figure 1.1).
3. V1 (First node) - Fully developed, unrolled leaves on the first trifoliate node (Figure 1.1).
4. V(n) stage- Fully developed trifoliate leaves on the nth node (Figure 1.1).
5. R1 (Beginning bloom)- Occurrence of a flower at any node (Figure 1.1).

6. R2 (Full bloom)- An open flower in one of the two uppermost nodes with a fully developed leaf (Figure 1.1).
7. R3 (Beginning pod)- At least one pod is 0.475 cm long in one of the four uppermost nodes with a fully opened leaf (Figure 1.1).
8. R4 (Full pod)- Pod length is 1.9 cm at least in one of the four uppermost nodes with a fully opened leaf (Figure 1.1).
9. R5 (Beginning seed)- Seed 0.32 cm long in a pod at one of the four uppermost nodes with a fully opened leaf (Figure 1.1).
10. R6 (Full seed)- Pod with at least one green bean in one of the four uppermost nodes with a fully opened leaf (Figure 1.1).
11. R7 (Beginning maturity)- One pod on the main stem reached its maturity showing a yellow to tan color (Figure 1.1).
12. R8 (Full maturity)- About 95% of the pods in the stem attain the mature tan color. Five to ten days are required for the pods to reach their harvest moisture if the weather is dry and warm (Figure 1.1).

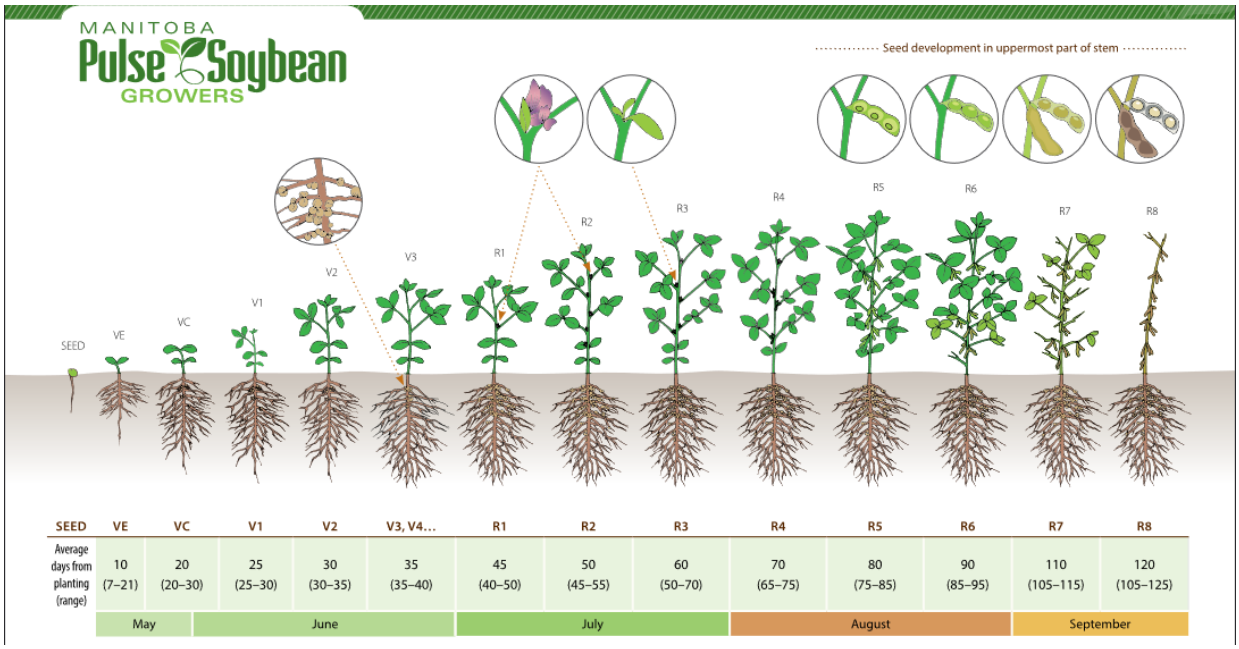

Figure 1.1: Vegetative and reproductive growth stages of soybean. VE to VN represents vegetative growth stages and R1 to R8 reproductive stages of soybean (*Glycine max* L.). (Image credit: Manitoba Pulse Soybean Growers)

Variety selection and adaptation

Soybeans are a photosensitive crop and respond to heat units. Thus, the crop period of a variety is highly dependent on the latitude of the area and is adapted to each narrow north-south zone. For North Dakota, maturity group (MG) 00 is desirable for the northern Red River Valley and the north central area. Maturity group 0 is commonly grown in many counties of North Dakota, while MG I is mainly for southeastern North Dakota.

Amidst the unpredictable weather patterns in recent years, it is better to select a high-yielding variety in a particular maturity group using the average yield data from several locations and several years. In addition, selecting a variety with resistance or tolerance to pathogens (e.g., *Phytophthora*) and other stressors (e.g., iron deficiency chlorosis) is important (Kandel and Endres 2023).

Soybean production

Seed quality is an essential factor to be considered while planting. High-quality seeds with disease package genetics matching to location need to be considered to ensure a good stand establishment. Seeds having the seed coat cracked are not ideal for planting. Moreover, since the size of the seeds influences the germination and early vigor of the seedlings, uniform seed size, with few small and large seeds, is ideal (Berglund et al. 1998).

Seedbed preparation

Although soybeans can be planted in a variety of soil types with suitable cultural and management practices, saline, waterlogged soils are not suitable for growth. Considering seed size and physiology, it is required for the seeds to have 50% of its weight in moisture to germinate'. Soybean are planted at a depth of 2.54 to 3.31 cm deep, which is why the crop requires firm and uniform seed bed for a better stand. In fields with no-till, special planters or drills may be required to handle surface crusts (Kandel and Endres 2023).

Planting date

Like any other summer crop, soybean is also susceptible to frost and hail injury. Planting is recommended when the soil temperature is at least 10ºC and the air temperature is favorable. Planting earlier in the season is highly vulnerable for the crop and this may cause damage to the seedlings by cold. Moreover, planting in wet and cool soil may result in low seedling stand and vigor. When the season is favorable, planting between May 10 to May 25 is ideal. The full-season soybean varieties (varieties that require a longer growing season to mature) may take full advantage of the season when the weather conditions promote growth, which yields more than short-season varieties. When the expected initial stand is not achieved, the decision regarding

replanting soybeans should be considered by comparing the yield of the replanted crop with the yield of the initial stand to offset the cost of replanting (Kandel and Endres 2023).

Planting rate

The planting rate influences the seedling establishment and yield of the crop. High rates in low rainfall areas may result in drought stress to the seedlings when compared to lodging in high rainfall areas. Too low of a rate promotes poor pod set and excessive plant branching. A desirable rate of 150,000 to 175,000 pure live seeds (PLS) per acre is recommended, according to research trials conducted by the North Dakota State University (NDSU). An average of 3,000 seeds per pound is recommended for a good stand establishment. Rather than targeting the desired range of plant rate, it might be economical to consider an extra 10 percent to overcome the loss of seedlings due to natural stress (Kandel and Endres 2023).

Row spacing

Producers in North Dakota follow narrow spacing (35.56 to 55.58 cm) based on the studies from NDSU. A study conducted by Endres et al. (2020) revealed that plants in narrow rows favor high yield if the initial stand is desirable and the weeds are not a concern.

Soil fertility

Soybean requires all major nutrients including Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Sulfur (S), Magnesium (Mg), Zinc (Zn), Copper (Cu), Manganese (Mn), Iron (Fe), Boron (B), Chloride (Cl), Nickel (Ni) and Molybdenum (Mo) for its growth (Kandel and Endres 2023).

Nitrogen

Soybeans meet the needs of nitrogen through a symbiotic association with bacteria living in the rhizosphere, *Bradyrhizobium japonicum* (Kirchner) Jordan (Buchanan 1980). In exchange with carbohydrates and minerals, this N-fixing bacteria provides the fixed nitrogen that can be

absorbed by the plant. So, in a field previously cultivated with soybean, no in-season nitrogen fertilizer application is recommended. Moreover, if excess nitrate is available in the soil, this may elevate the incidence or severity of iron deficiency chlorosis. However, if soybean is introduced to a field for the first time, the seeds are recommended to inoculate with granules, peat, or liquid-based *B. japonicum* inoculum (Kandel and Endres 2023).

Phosphorus

Broadcast application of phosphorus is desirable only when the soil level is low to very low (less than 8 mg/L). Since the soybean is excellent in utilizing carryover fertilizer, in-season fertilizer application is not recommended if the soil level is medium or higher (Kandel and Endres 2023).

Potassium

Among different soil textures, coarse textures are more vulnerable to potassium deficiency than heavier soils. If the soil potassium level is less than 120 mg/L and it has a clay mineral index (smectite/illite (S/I) ratio) less than 3.5, 27.2 kg per acre of potassium oxide (K_2O) is recommended. Broadcast or banded application can be followed; however, the fertilizer should not be applied along with the seed (Kandel and Endres 2023) .

Sulfur

In comparison with corn (*Zea mays* L.) and small grains, soybeans suffer a low risk of sulfur deficiency. It is desirable to scout the field looking for symptoms of S deficiency, which is pale green leaves with prominent veins without necrosis. Field need-based applications are desirable (Kandel and Endres 2023).

Zinc

The zinc level in soils of North Dakota is not sensitive to soybean compared to Zn-sensitive crops such as dry bean (*Phaseolus vulgaris* L.), corn (*Zea mays* L.), flax (*Linum usitatissimum* L.) and potato (*Solanum tuberosum* L.) (Kandel and Endres 2023).

Iron

The soil with high pH (more bicarbonate) and wet conditions is more susceptible to iron deficiency chlorosis (IDC). Integrated management practices should be adopted to manage IDC, which includes selecting a field that is less prone to IDC and using an IDC-tolerant variety. A suitable crop rotation that balances the salinity of the soil might also be included. A foliar spray of Iron is not effective, but in-furrow application of ortho-ortho-EDHHA Fe chelate with water is recommended during planting (Kandel and Endres 2023).

Irrigation

Adequate water supply from the beginning bloom to the seed fill stage influences the number of pods, the number of seeds per pod, and the weight of the seeds. If a dry spell occurs during the planting through vegetative stages, irrigation is necessary for soybean establishment and growth of the plant, but it does not necessarily determine the yield (Heatherly 1998).

Soybean disease identification and management

Like any other stress factors, diseases have the potential to impact soybean yield and grain quality. Over the past five decades, soybean diseases alone caused estimated yield losses of hundreds of thousands of million dollars (Bradley et al. 2021; Doupnik 1993, Koenning and Wrather 2010, Wrather et al. 1995, Wrather and Koenning 2006). The most common diseases observed in both northern and southern soybean growing regions in the U.S. are soybean cyst nematode, charcoal rot, and seedling diseases (Roth et al. 2020). However, depending on the environmental factors favorable for the pathogen, sudden death syndrome and Sclerotinia stem rot

(white mold) are also considered serious threats to soybean production (Roth et al. 2020). An increase in frequency in the occurrence of *Diaporthe* diseases, frogeye leaf spot, and root-knot nematodes has also been reported (Roth et al. 2020).

Seedling diseases

Soil-borne pathogens such as those belonging to the *Fusarium, Phytophthora, Pythium,* and *Rhizoctonia* genera tend to cause infection to the seedlings before and after emerging from the soil.

Species of *Phytophthora* and *Pythium* may rot the seeds before emergence, cause pre- and post-emergence damping off, and soft brownish water-soaked rot on roots (Figure 1.2A and B). The transport of water and nutrients is severely affected and may result in chlorosis of leaves during crop growth stages V1 and V2. Later, the plants may wilt and die. The wet and cool environment that persists during the first few weeks of planting promotes oomycete diseases in seedlings (Giachero et al. 2022, Schmitthenner 2000).

Species of *Rhizoctonia* produce rusty, brown-colored lesions on hypocotyl near the soil line (Figure 1.2C) and although the plant surpasses the infection during the seedling stage, they may be stunted (Markell and Malvick 2018, Roth et al 2020). Warm wet soil with high organic matter and plant stress due to physical or chemical injury may favor infection (Markell and Malvick 2018). The disease tends to cause significant stand reductions, so growers might be forced to replant the crop. Scouting the fields during early vegetative stages is recommended for the possible identification of any problem regarding plant stand. Stand reduction may also be attributed to any abiotic factors other than diseases (Kandel and Endres 2023), which may be diagnosed during a soil sample test in laboratories.

Species of *Fusarium* infect the root system (Figure 1.2D) and cause yellowing of the leaves, which starts from the leaf margins. Sometimes, the pathogen remains latent within the host and may cause pod abortion during the host's reproductive stages (Markell and Malvick 2018; Winsor 2020).

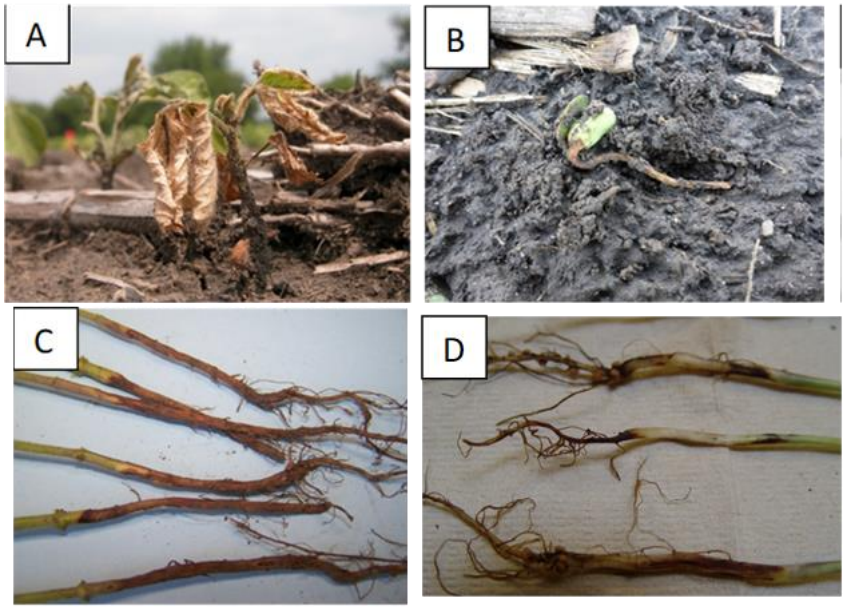

Figure 1.2: Damping off due to infection by *Phytophthora sojae* (A), rotted seedling root from *Pythium* infection (B), and sunken, dry lesions near soil line due to *Rhizoctonia solani* infection (C), and soybean seedlings infected with *Fusarium* spp. (Image credits: Anonymous 2019 h, i, k, and m)

Commercial cultivars with resistance to species of *Fusarium*, *Pythium*, and *Rhizoctonia* are not available. However, breeding efforts have identified genes in soybeans that confer partial resistance to *Phytophthora sojae* Kaufmann and Gerdemann (Dorrance et al. 2003). Cultural practices, such as proper drainage and tillage, help in controlling diseases. Rotating soybeans with corn may not contribute to disease management directly since corn is an alternate host to these pathogens (Rojas et al. 2019). However, an extended crop rotation including diverse crops may be recommended considering their market values (Leandro et al. 2018, Rojas et al. 2019). Treating

seeds with ethaboxam (Fungicide Resistance Action (FRAC) group U), mefenoxam (FRAC 4) or oxathiapiprolin (FRAC F9) containing products are effective in controlling multiple species of *Phytophthora* and *Pythium* (Scott et al. 2020; Wang et al. 2023).

Stem diseases

Stem diseases of soybean have caused yield losses of 0.16 million metric tons in the year 2022 in the U.S (Allen et al.2023). The most common pathogens causing stem diseases are *Cadophora gregata* (Allington & Chamberlain) Harrington and McNew, comb. nov, *Colletotrichum truncatum* (Schw.) Andrus and Moore and other *Colletotrichum* spp., *Diaporthe* Nitschke [= *Phomopsis* (Sacc.) Bubak] spp., *Macrophomina phaseolina* (Tassi) Goid, and *Sclerotinia sclerotiorum* (Lib.) DeBary:

1. Brown Stem Rot (*Cadophora gregata*): In early summer, this soil-borne fungus enters the host through roots and colonizes inside the stem-producing conidia. No visible symptoms are observed on the infected plants (Grau and Heimann 1982). The stems must be split to see the browning of the pith region and vascular system (Figure 1.3A) (Grau and Heimann 1982, Chamberlain and Bernard 1968). In some cases, interveinal chlorosis and necrosis of leaves may be observed, which may often be confused for sudden death syndrome caused *by Fusarium virguliforme* O'Donnell & Aoki (Markell and Malvick 2018).

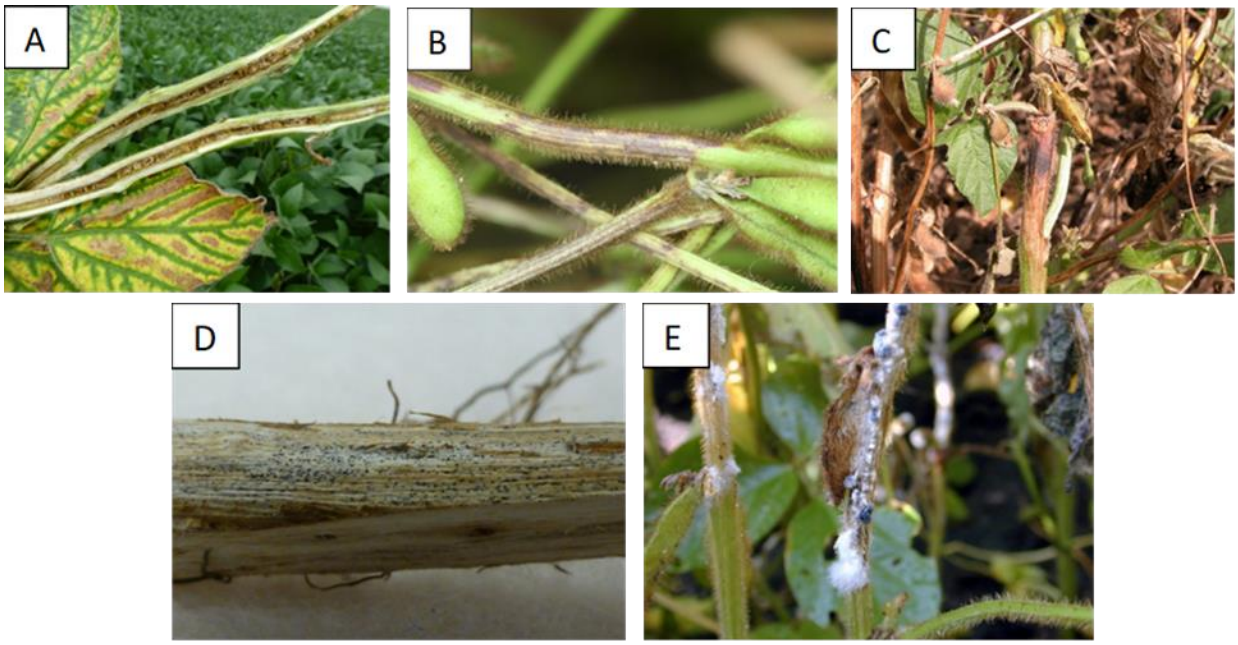

Figure 1.3: Foliar and stem symptoms characteristic to brown stem rot(A), stem lesions caused by anthracnose disease on soybean stem (B), reddish brown canker symptom near the node of the soybean stem characteristic to soybean stem canker(C), numerous grey colored micro sclerotia on the stem infected by *Macrophomina phaseolina* (D), and white fluffy growth and sclerotia on the soybean stem infected by *Sclerotinia sclerotiorum* (E). (Image credits: Anonymous 2019 a, c, e, n and p)

2. Anthracnose (*Colletotrichum* spp.): Seedlings exhibit pre- and post-emergence damping off while lesions can be observed on the cotyledons. At later growth stages, irregular, dark to pale brown blotches can be seen on the stem, pods, and petioles (Figure 1.3B). The leaves may roll up, and premature defoliation has also been reported. The asexual fruiting bodies of the pathogen, acervuli, may be seen as concentric rings on the lesions or blotches when viewed closely. During the reproductive stages of the plant, the pods may get twisted and aborted. If the seeds are infected, it will result in a poor germination rate (Dias et al. 2016; Markell and Malvick 2018; Yang and Hartman 2015).
3. Stem canker (*Diaporthe aspalathi* Jansen, Castl. & Crous and *D. caulivora* [Athow & Caldwell] Santos et al): Infection usually occurs during early reproductive stages.

Reddish brown sunken lesions are seen on the stem, particularly near to the node region (Figure 1.3 C). The lesion is characterized by green tissues, both on the upper and lower portion of the stem. Lesions coalesce to girdle the stem and eventually plants may die. Interveinal chlorosis may also occur before the plant death and the dried leaves appear to be attached to the leaf petiole on the stem (Anonymous 2019n).

4. Charcoal rot (*Macrophomina phaseolina* [Tassi] Goid): In most cases even if the infection happens at an early stage, the symptoms are not observed until the flowering growth stage (Markell and Malvick 2018, Mengistu et al. 2011). The disease initiates with a grey discoloration on the lower stem and tap roots. Premature yellowing and wilting of the plants follow. Plants may die with attached dried petioles on the stem (Mengistu et al. 2011). The surviving structures of the fungus, called microsclerotia, may be visible on close inspection under the epidermis of the stem (Figure 1.3 D) or roots. The disease frequently occurs in patches, especially targeting drier spots in the field. At times, the damage by the disease is misdiagnosed as drought damage, however, the plants die more rapidly when infected with *Macrophomina phaseolina* than without the disease (Romero et al. 2017; Markell and Malvick 2018).
5. White mold (*Sclerotinia sclerotiorum*): The disease is typically observed during the reproductive growth stages of the plant (Dorrance and Novakowiski 2008). The leaves show grey discoloration, which turns brown and the plant wilts gradually. The disease becomes obvious when one or two plants die out of a healthy canopy during the late season. When the lower part of the stem of an infected plant is inspected, a bleached area may be visible. During high moisture conditions, this area may get covered with white fluffy mycelia of the pathogen (Figure 1.3 E). The bleached area can extend in

both directions of the stem. Later, black-colored, oblong-shaped resting structures of the fungus, and sclerotia can be seen on these lesions (Dorrance and Novakowiski 2008). The sclerotia is also produced inside the stem. If the infection occurs during pod development stages, the seeds may get diseased and become flat, shriveled, and may be replaced with sclerotia (Dorrance and Novakowiski 2008).

Foliar diseases

Foliar diseases affect the capacity of the leaves to absorb nutrients and engage in photosynthetic processes which may induce necrosis. Infection which may start in the foliage may spread to the pods infecting seeds as well. Major foliar diseases of soybean are:

1. Bacterial Blight (*Pseudomonas savastanoi* pv. *glycinea*): Small water-soaked lesions appear on the leaves in the upper canopy. Later, the center of these lesions turns brown, and a yellow halo is produced surrounding the lesion. The spots may coalesce and result in a tattering of leaves (Figure 1.4A). (Markell and Malvick 2018).
2. Cercospora leaf blight (*Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) Gardner): The leaves in the upper canopy turn yellow, change later to purple to brown color (Fig. 1.4 B). Necrosis happens on the leaf tissue which eventually coalesces and results in the dropping of the leaves (Hershman 2009, Markell and Malvick 2018).
3. Frogeye leaf spot (*Cercospora sojina* Hara): In the beginning stages of the infection, dark spots appear on the leaves. A purple ring arises around the spots and the center of the spots may turn brown. These spots may coalesce, form lesions, and destroy large portions of the leaf tissue (Fig 1.4 C) (Cruz 2008; Markell and Malvick 2018).
4. Sudden death syndrome (*Fusarium virguliforme* O'Donnell & Aoki): Lateral and tap roots would be rotted. Due to the transport of toxins to upper plant parts, yellow spots

are observed between veins of the leaves during the initial stages of infection. Later, the spots become large and necrotic, leaving the midrib and veins of the leaves green (Fig. 1.4 D). Leaves eventually drop with petioles still attached to the stem. Blueish fungal growth might be observed on the surface of the roots when the soil moisture is high (Anonymous 2019o).

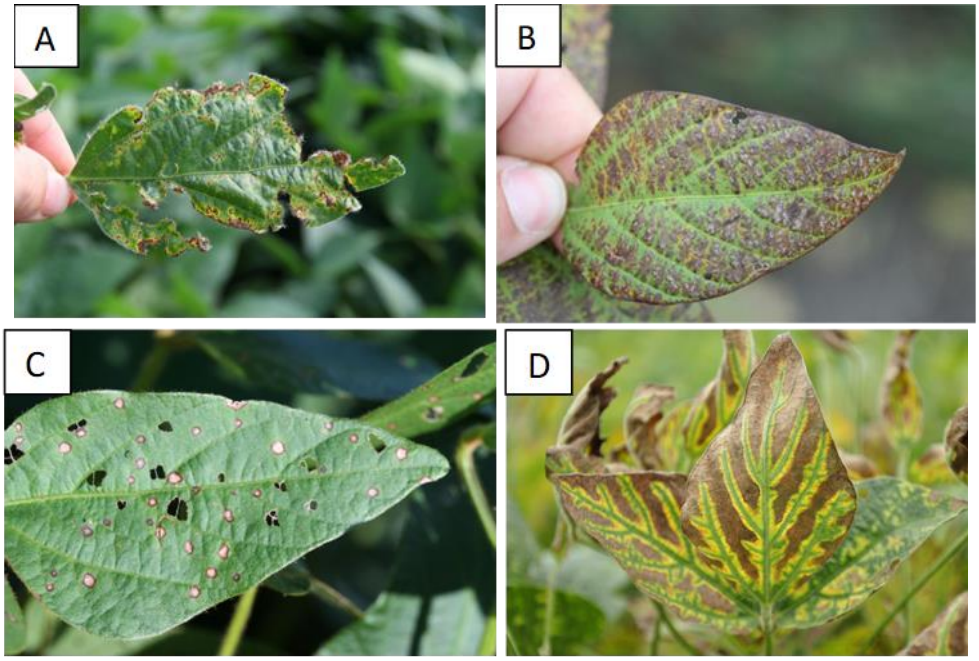

Figure1.4: Tattering of leaves when leaf spots caused by *Pseudomonas savastanoi* pv. *glycinea* coalesce (A), leaf blight caused due to *Cercospora kikuchii* infection on soybean leaf (B), reddish brown margins around gray centered mature spots due to frogeye leaf spot (C), and necrotic blotches between veins of the leaves characteristic to sudden death syndrome (D). (Image credits: Anonymous 2019b, d, g and o)

Seed diseases

1. Purple seed stain (*Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) Gardner)*:* The disease is favored by wet and warm weather conditions during and after the flowering stage of the crop. The infected seeds may appear as symptomless or purple or pink stain starting from the hilum (Fig 1.4 A). The yield is not affected however the

cropvalue may be reduced due to dockage or denial of seed certification (Anonymous 2019l).

2. Diaporthe (Phomopsis)Seed Decay: The disease is primarily caused by *Diaporthe longicolla* (Hobbs) Santos et al. and other species of *Diaporthe* (Petrovic et al. 2021). The disease development is favored by warm and humid conditions, which may be prevalent when the crop is at R6 (full seed) to R8 (full maturity) growth stages. Seeds appear to be shriveled, chalky and cracked. Sometimes, when the infection is high, white fungal growth can also be observed (Fig 1.4 B). When the infected seeds germinate, the seedlings may have reddish-brown pinpoint lesions obvious just above the soil line (Anonymous 2019f).

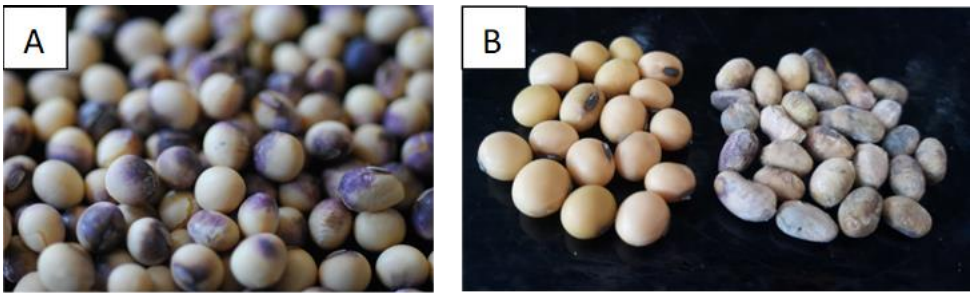

Figure 1.5: Purple stain symptoms on soybean seeds caused by *Cercospora kikuchii* (A), and small, elongated, wrinkled, chalky seeds covered with mycelia due to infection of *Diaporthe* spp. on soybean seeds (right) compared with healthy seeds (left)(B). (Image credits: Anonymous 2019 f and l)

General disease management practices for soybean

1. Crop rotation: Rotating soybeans with non-host crops can help break the disease cycle and reduce pathogen populations in the soil. This strategy is particularly effective for soilborne diseases. For example, rotating soybean with small grain crops such as fescue (*Festuca arundinacea* Schreb), sorghum (*Sorghum bicolor* (L.) Moench) and

wheat (*Triticum aestivum* L. em Thell) can help manage diseases like soybean cyst nematode (SCN) and sudden death syndrome (SDS) (Rupe et al.1997).

2. Resistant varieties: Planting resistant soybean varieties is a crucial strategy for disease management. Resistant varieties can effectively reduce the impact of many diseases. For example, resistant varieties are available for managing diseases like SCN, soybean rust, and Phytophthora root and stem rot (Roth et al. 2020).
3. Seed treatment: Treating soybean seeds with fungicides can protect young seedlings from soilborne and seed-borne pathogens. Seed treatments can provide an initial defense until the plants establish themselves (Wise et al. 2018).
4. Cultural practices: Implementing good cultural practices can help reduce disease incidence. These include proper planting dates, optimizing plant spacing, and maintaining optimal fertility levels. Proper weed control is also important, as weeds can serve as alternative hosts for diseases (Anonymous 2019e, j and n).
5. Foliar fungicide application: Foliar fungicides can be used to manage foliar diseases in soybeans when disease pressure is high. Fungicide application should be based on disease scouting (Brown et al. 2024) and thresholds (if available), and the choice of fungicide should consider disease resistance management. The recommendations on the label of the fungicide product should be considered regarding timing and the frequency of the spray.
6. Integrated Disease Management (IDM): Adopting an integrated approach that combines multiple strategies, such as crop rotation, resistant varieties, cultural practices, and targeted fungicide application, is crucial for effective disease

management. IDM emphasizes the use of multiple tools to minimize disease impact while reducing reliance on any single strategy (Anonymous 2019e, j and n).

Diaporthe associated diseases

Diaporthe diseases, which caused 0.2 million metric tons loss in 2022 considered to be important emerging diseases of soybean (Allen et al. 2023). Species of *Diaporthe* belonging to phylum Ascomycota, are reported as endophytes, and saprobes (Gomes et al. 2013). These organisms are known to infect several hosts, which include soybean (Zhang et al. 1998, Santos et al. 2011, Petrovic et al. 2021), sunflower (*Helianthus annuus* L.) (Yang et al. 1984; Mathew et al. 2015), multiple species of *Citrus* (Huang et al. 2015), almond (*Prunus dulcis* Mill Webb.), peach (*Prunus persica* Batsch.) (Diogo et al. 2010) and grapes (*Vitis vinifera* L.) (Baumgartner et al. 2013) among other crops. On soybeans, species of *Diaporthe* are known to cause stem canker, pod, and stem blight, and seed decay in several production regions around the world (Lehman 1923, Sinclair 1993, Pioli et al. 1997).

Historically, the type of host, disease symptoms, colony appearance, presence of perithecia, presence of anamorph, and presence of alpha- and beta-conidia were used for the identification of *Diaporth*e at the species level (Santos et al. 2011). However, due to the variability among inter- and intraspecific isolates, the morphological identification at the species level is not reliable (Santos et al. 2011). Since the 1990s, sequence comparison of gene regions such as Internal Transcribed Spacer region (ITS), translational elongation factor-1 alpha (TEF1-α), β-tubulin, calmodulin, and actin are widely used for species recognition (Santos et al. 2011, Udayanga et al. 2015, Hosseini et al. 2020).

Stem canker

Species of *Diaporthe,* such as *D. caulivora* (syn. *D. phaseolorum* var. *caulivora*. [Athow & Caldwell]), and *D. aspalathi* (syn. *D. phaseolorum* var. *meridionalis* Fernandez) cause northern stem canker and southern stem canker, respectively (Backman et al. 1985; Udayanga et al. 2015). Symptoms are seen as reddish-brown lesions near the base of the leaf petiole, which enlarge to form sunken cankers with reddish margins near the nodes. The lesions may coalesce, leading to girdling and kill the plant. During the late season, the diseased plants may appear dead attached to dried leaves. The death of the upper four to six internodes of the plant may also occur. Due to the transport of fungal toxins through the vascular system, interveinal chlorosis and necrosis may be observed, which may be misdiagnosed as other foliar infections (Backman et al. 1985). *Diaporthe aspalathi* and *D. caulivora* overwinter in infested crop debris and soil. An extended period of wet and warm weather favors the production of conidia and/ or ascospores which are disseminated through rain splashes. The infection happens during the early vegetative growth stages of the crop however the symptoms start to develop during the reproductive growth stages of the crop (Anonymous 2019n). Management practices for the disease include choosing cultivars resistant to these pathogens, crop rotation, and tillage (Anonymous 2019n).

Pod and stem blight

This was the first *Diaporthe* disease reported in soybeans in the U.S. (Lehman 1923). This disease is primarily caused by *Diaporthe sojae* Lehman (syn. *D. phaseolorum* var. *sojae* (Lehman) Wehmeyer) and *D. longicolla* (syn. *Phomopsis longicolla* Hobbs) (Anonymous 2019j, Lehman 1923, Santos et al. 2011) and is identified by the appearance of black colored linear raised specks on the mature soybean stems, pods and seeds (pycnidia produced by the fungus) when the plant reaches R6 (full seed) to R8 (full maturity) growth stages. Mature pods with

pycnidia often carry cracked and shriveled seeds, which may have a low germination rate. The seedlings grown from these infected seeds may lose their vigor (Anonymous 2019j, Lehman 1923, Markell and Malvick 2018).

These organisms may overwinter in the crop residues and soil. Infected seeds serve as another source of inoculum for the disease. Weeds such as *Amaranthus palmeri* (pigweed) and *Abutilon theophrasti* (velvetleaf plant) can act as alternate hosts to the pathogens. Rain during the early vegetative growth stage of the crop helps in spreading the spores of the fungus from infected crop residues and seeds. But the host becomes susceptible only when it reaches between R5 (beginning seed) and R6 (full seed) growth stages. When the soybean reaches the R7 (beginning maturity) growth stage, seed moisture drops resulting in low infection. However, when favorable conditions such as wet and warm weather occur, the infection level progresses (Anonymous 2019j).

Pod and stem blight is often misdiagnosed as other diseases such as anthracnose (*Colletotrichum* spp.) and charcoal rot (*Macrophomina phaseolina*). Thus, to distinguish pod and stem blight from other diseases, one should look for the presence of linear pycnidia, which is the primary diagnostic sign of species of *Diaporthe* and is favored by warm and wet weather conditions (Anonymous 2019j).

Planting pod and stem blight-resistant varieties help in reducing the incidence of the disease. Cultural practices such as rotating soybeans with non-host crops such as corn or wheat can reduce the inoculum load in the field. Seed treatment with fungicides is recommended, and it usually increases seed emergence. Delayed harvest due to late-season rains is not recommended since the plants would be likely exposed to warm and wet conditions favoring disease development (Anonymous 2019j).

Diaporthe (Phomopsis) seed decay

Although multiple species of *Diaporthe* infect soybean seeds, *Diaporthe longicolla* is the predominant species causing seed decay (Petrovic et al. 2021). The economic loss caused by the *Diaporthe* seed decay reported as 0.1 million metric tons ($14.9 billion loss) in 2022 in the U.S exposes the potential revenue loss that may occur to the soybean production industry (estimated total revenue from soybean production in the year 2022 in the U.S is 634.98 billion dollars (United States Department of Agriculture- National Agricultural Statistics Service (USDA-NASS 2023), Anonymous 2019f).

The symptoms may range from absent to severe. Infected seeds appear as shriveled, and cracked, and may display white fungal growth when the pods are opened. Low germination or delayed germination of infected seeds may also occur. When the infected seeds germinate, reddish-brown point lesions may be observed on the cotyledon or the stem near the soil line. Since the pathogen can disseminate the infection to the next generation of plants, the control of the disease is vital (Anonymous 2019f, Shortt et al. 1981, Sinclair 1993).

Adopting varieties with resistance to the causal organisms is the best option to manage the disease. Cultural practices such as conventional tillage and crop rotation with non-host crops such as corn are beneficial in reducing the inoculum in the field. Prophylactic application of foliar fungicides between R3 (beginning pod) and R5 (beginning seed) growth stages may reduce seed infection, especially in seed production fields.

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CHAPTER TWO: MONITORING SENSITIVITY OF SPECIES OF *DIAPORTHE* TO AZOXYSTROBIN FUNGICIDE IN SOYBEAN (*GLYCINE MAX* L.)[1]

Abstract

Species of *Diaporthe* associated with pod and stem blight, seed decay, and stem canker in soybean (*Glycine max* L.). caused a total estimated yield loss of 0.2 million metric tons in 2022 in the U.S. Among the fungicide products labeled to provide protection against *Diaporthe* species, those containing quinone outside inhibitor (QoI) are considered high risk for fungicide resistance. The objective of this study was to determine the sensitivity of isolates of *D. aspalathi*, *D. caulivora* and *D. longicolla* to azoxystrobin fungicide. A total of 75 isolates obtained from 16 U.S. states were evaluated using mycelial growth inhibition assays on 2% water agar amended with azoxystrobin concentrations of 0, 0.001, 0.01, 0.1, 1.0, and 10.0 µg/mL. Salicylhydroxamic acid (20 µg/ml) was added to the fungicide media to inhibit alternative fungal respiration pathways. The experiment was performed in a completely randomized design with four replications and repeated once. Five to eight days post-inoculation, the effective concentration (EC) needed to inhibit 50% (EC_{50}) of the mycelial growth was determined. A significant effect of isolates was observed on EC_{50} of *D. aspalathi* (*P*=0.002), *D. caulivora* (*P*=0.014), and *D. longicolla* (*P*=0.003). A significant difference in EC_{50} (expressed as Relative Treatment Effect) was observed among isolates of *D. aspalathi*. The results suggest azoxystrobin is effective in controlling *Diaporthe* diseases in soybean. Future studies should include continuing further to monitor *Diaporthe* populations for both changes in the efficacy of QoI fungicides and mutations associated with fungicide resistance.

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Introduction

Species of *Diaporthe* are known to cause various diseases in soybean (*Glycine max* L.), including stem canker (*D. aspalathi* Jansen, Castl. & Crous and *D. caulivora* [Athow and Caldwell] Santos et al.), pod and stem blight (*D. sojae* Lehman and *D. longicolla* Santos et al.), and Diaporthe seed decay (*D. longicolla* Santos et al. and other species of *Diaporthe*). These diseases have resulted in significant yield losses, amounting to estimated losses 0.2 million metric tons in 2022 (Allen et al. 2023).

Currently, disease management strategies primarily involve crop rotation with non-host crops and tillage practices (Anonymous 2019a, b, c). While sources of resistance have been identified for *D. aspalathi, D. caulivora*, and *D. longicolla*, it remains unclear if these genes have been incorporated into commercially available cultivars (Ghimire et al. 2019). There are several factors that may hinder the incorporation of a resistance gene from a soybean genotype into commercial varieties. These include limited genetic diversity, linkage drag, and lack of validation in diverse environments (Cui et al.2022, Kueneman 2022). Furthermore, even though parent materials for developing resistant cultivars are available in the United States Department of Agriculture (USDA) Soybean Germplasm collection, the reaction of *Diaporthe* to soybean depends on the isolate virulence and/or aggressiveness (Mohan et al. 2023). Additionally, the efficacy of foliar fungicides against species of *Diaporthe* is not well understood (Batzer and Mueller 2020, Berkland 2011, Wrather et al. 2004). In the study conducted by Batzer and Mueller (2020), an increase in prevalence and dominance of endophytic *Diaporthe* spp. was observed in stems, leaves, and seeds of soybean in plots treated with a mix of fluxapyroxad (FRAC 7) and pyraclostrobin (FRAC 11) fungicides compared to the control treatment. Wrather et al. (2004) showed an increase in percent of seeds infected with *Diaporthe* in azoxystrobin (FRAC 11)

treated plots at R3 (beginning pod), R6 (full seed) and R3 (beginning pod) +R5 (beginning seed) growth stages of soybean (*Glycine max* L.). In contrast, the research by Berkland (2011) reported a decline in the *D. longicolla* infected soybean seeds on application of pyraclostrobin at pod set growth stage by 1.9% The possibility of development of QoI fungicide resistance among *Diaporthe* population was speculated as a reason for the predominance of *Diaporthe* spp. in plots treated with the quinone outside inhibitor (QoI) (FRAC 11) fungicides in soybean in Batzer and Mueller (2020).

According to the data from the United States Department of Agriculture-National Agricultural Statistical Service (USDA-NASS) from 2013 and 2021 surveys, fungicide use accounted for 11% of all chemicals used in soybean cultivation in 2012, and that number doubled by 2020 (USDA-NASS 2013 and 2021). This suggests an increasing trend among farmers toward the use of fungicides, driven by their prophylactic ability to mitigate diseases and promote plant health, which are recommended at the R3 (beginning pod) growth stage of soybean (Floyd et al.2021). Particularly, certain chemicals like azoxystrobin, pyraclostrobin, and trifloxystrobin of the QoI family, classified under the Fungicide Resistance Action Committee (FRAC) group 11, have gained popularity (Phillips et al. 2021). These chemicals act by interfering with the electron flow at the quinone outside site of the bc1 complex (complex III) within fungal cell mitochondria, effectively inhibiting fungal respiration (FRAC 2023).

The most common mechanism of resistance observed in QoI-resistant fungal individuals is a single-site mutation, resulting in the substitution of the amino acid glycine (G) with alanine (A) at position 143 (G143A) in the cytochrome b (cyt *b*) gene region (Vincelli 2002). Among soybean pathogens, reports have shown that *Corynespora cassiicola* (Berk. and Curt.) Wei (Rondon and Lawrence 2019), *Cercospora sojina* Hara (Standish et al.2015), and *Septoria glycines* Hemmi

(Neves et al. 2022) have developed G143A mutation. The single amino acid mutation substituting phenylalanine (F) with lysine (L) at 129th codon of cyt *b* gene (F129L) reported in *Alternaria solani* (Ellis and Martin) Sorauer in potato (*Solanum tuberosum* L.), *Cercospora beticola* Sacc. in sugar beet (*Beta vulgaris* L.) and *Phakopsora pachyrizhi* Sydow (Klosowoski et al. 2016) in soybean contributes to the partial resistance to QoI. Moreover, least common single site mutation substituting glycine (G) by arginine (R) at 137th codon of cyt *b* gene (G137R) reported in *Magnaporthe oryzae* BC Couch in rice (*Oryza sativa* L.) (Miao et al. 2020) also confers resistance to QoI. A study conducted by Floyd and Malvick (2022) examined the sensitivity of 11 isolates of *D. caulivora* isolates and 13 of *D. longicolla* from various counties of Minnesota to pyraclostrobin (Headline®) and the isolates were determined to be sensitive. Since Floyd and Malvick (2022) included isolates from Minnesota only, it is important to monitor the fungicide sensitivity of *Diaporthe* isolates from other soybean production regions in the U.S.

Thus, the objective of the study was to evaluate the *invitro* sensitivity of isolates of *D. aspalathi, D. caulivora* and *D. longicolla* to azoxystrobin (QoI) fungicide in soybean.

Materials and methods

Selection and identification of isolates

Isolates of *D. aspalathi* (*n*=15), *D. caulivora* (*n*=19) and *D. longicolla* (*n*=41) were collected from Alabama (*n*=1), Delaware (*n*=2), Georgia (*n*=1), Illinois (*n*=1), Indiana (*n*=10), Iowa (*n*=4), Kentucky (*n*=16), Michigan (*n*=5), Minnesota (*n*=2), Mississippi (*n*=4), Missouri (*n*=2), New York (*n*=1), North Dakota (*n*=1), South Dakota (*n*=14), Tennessee (*n*=1), and Wisconsin (*n*=10).

Isolates of *Diaporthe* used in this study were recovered from soybean plant parts exhibiting stem canker symptoms, such as reddish-brown discoloration and/or pycnidia on the

stem, or from asymptomatic seeds collected from the plants with linear black lesions on the stems, collected from commercial fields of 16 U.S states between 2014 and 2022. The plant parts were processed and the fungal isolations were done from cut stem pieces (1 cm long) after being subjected to surface sterilization with 0.05% sodium hypochlorite for 1 min, 70% ethanol for 1 min, washed twice with sterile water, and then air-dried on filter paper in a laminar air flow hood. Three stem pieces from each plant were plated on full-strength Potato Dextrose Agar (PDA) media amended with 0.3g/L of streptomycin sulfate and incubated at 23±2ºC for 10 days under 12 hours of alternating light and dark conditions. Isolates emerging from plant parts were recovered by hyphal tipping from the leading colony edge and transferred to fresh PDA plates. The fresh plates were incubated under the same conditions described previously. The cultures were observed under a microscope for identification of colony growth, pycnidia and/or perithecia development, and conidia formation characteristic of *Diaporthe* species in soybeans (Petrovic et al.2021, Udayanga et al.2015).

For molecular identification, the mycelia from 7-day-old cultures were harvested and ground with a spatula sterilized with 70% ethanol in 700µL of the lysis buffer (1 M of Tris-HCl, 0.5 M of EDTA, 5 M of NaCl, and 10% sodium dodecyl sulfate) in a 1.5mL Eppendorf tube. Then the tube was stored in a heating block at 65ºC for 15 minutes. Then, 150 µL of potassium acetate (60 ml of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of distilled water) was added to the tube. Later, the tubes were subjected to centrifugation at 18,000 rpm for 3 min. The supernatant was transferred to a new 1.5mL Eppendorf tube and an equal volume of isopropyl alcohol was added to the supernatant. In the sequence, it was centrifuged at 15,000 rpm for 1 min, followed by washing the pellet with 500µL 70% ethanol. The pellet was then centrifuged to 10,000 rpm for 1 min and air dried. To dilute the DNA precipitate, a 20µL Tris-

EDTA (10 mM of Tris and 1 mM of EDTA) was added and the quality of the DNA was checked using a Nanodrop spectrophotometer.

The extracted DNA was subjected to quantitative Polymerase Chain Reaction (qPCR) for identity confirmation of *D. aspalathi, D. caulivora* and *D. longicolla* using DA primer-probe pair (Mohan and Mathew *unpublished*), PL-3 primers-probe pair and DPC-primers- probe set, respectively (Kontz et al. 2016). For qPCR assays, the Applied Biosystems QuantStudio 3 and 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) was used. The qPCR mixture contained 10 µL of TaqMan® Universal Master Mix II (Applied Biosystems), 0.4µL each of forward and reverse primers, 0.2µL of fluorescent TaqMan® probe, and 7 µL of sterile nuclease-free water for each sample of DNA diluted to 2 ng/µL. The qPCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (Mohan and Mathew *unpublished*; Kontz et al. 2016).

***In vitro* sensitivity assay**

A total of 75 isolates belonging to *D. aspalathi, D. caulivora,* and *D. longicolla* were used to evaluate their sensitivity to azoxystrobin (96% active ingredient (a.i), Anonymous 2023. Syngenta United States). With the technical grade azoxystrobin (96% a.i), a stock solution of 100 mg/L was made by mixing 104 mg fungicide with 1 mL of acetone. A serial dilution technique was employed to make fungicide concentrations of 0.001,0.01,0.1,1 and 10 mg/L in acetone and added to 2% water agar, which was cooled to 55°C after autoclaving. Considering salicylhydroxamic acid (SHAM) as an inhibitor to the alternate respiration of the fungi in the sensitivity assays (Kashyap 2022; Shi et al. 2020), a final concentration of 20 µg/ mL of SHAM was made with methanol and added along with each fungicide concentrations except control

treatment. The concentration of 20 µg/mL SHAM was used since it did not affect the EC_{50} of *D. helianthi* and *D. gulyae* upon the addition of pyraclostrobin (Kashyap 2022).

A mycelial plug of 6 mm in diameter was made from the leading edge of the 7-day-old fungal colony and was transferred to the center of the media plate (90mm×90mm). The control treatment of each *Diaporthe* species was examined visually for their growth at five, eight and 10 days after inoculation to determine the days of incubation for each species. For five isolates each of *D. aspalathi, D. caulivora,* and *D. longicolla,* the control plates were incubated at 23ºC for five days, eight days, and ten days in the dark. Isolates of *D. aspalathi* did not grow at 23ºC for five, eight, and ten days however when they were incubated at 25ºC for five, eight and ten days, the isolates were fully grown over plates at eight days after inoculation. For *D. caulivora* and *D. longicolla*, isolates in control plates were fully grown at 5 days at 23ºC. After eight days and five days post inoculation for isolates of *D. aspalathi*, and *D. caulivora,* and *D. longicolla*, respectively, the mycelia grew over the edges of the control treatment plates. Thus, the isolates were determined to incubate in the dark at 23ºC for five days for *D. caulivora* and *D. longicolla,* and at 25ºC for eight days for *D. aspalathi*.

After the incubation period, the diameter of the mycelial growth was measured at right angles across the plates. The diameter of the plug was subtracted before averaging the mycelial diameter. For calculating the effective fungicide concentration at which the fungal growth was inhibited by 50% over non-treated control (EC_{50}) (Fungicide Resistance Action Committee 2023), the average mycelial growth was converted to percent mycelial growth inhibition by the formula, [100× ((average diameter of mycelia in control treatment – average diameter of mycelia in fungicide amended plate))/ average diameter of mycelia in control treatment]. EC_{50} was

determined using '*drc*' package (Ritz and Streibig 2005, Noel et al.2018) in R software (v4.1.1; R core team 2021; https://www.posit.com) using a four-parametric log-logistic model as follows:

$$f(x) = c + \frac{(d-c)}{1+\exp(b\log(x)-\log(e))} \quad (1)$$

where, d = lower asymptote, c = upper asymptote, b = slope, e = effective control to 50% growth inhibition (EC_{50}), and x = fungicide dosage (Noel et al. 2018).

In our study, we investigated the effect of SHAM at 20 and 100ppm concentrations on percent mycelial inhibition growth of selected isolates of *D. aspalathi* (16-OP-SB-DIA-035 and 16-OP-SB-DIA-041), *D. caulivora* (14-OP-PHO-SD-29 and 17-OP-DIA-SOY-029) and *D. longicolla* (14-PHO-SD-19 and 16-OP-SB-DIA-064). Media with no acetone and no SHAM acted as control treatment. Welch's two sample t-test was performed to compare the percent mycelial growth inhibition between treatments of 20ppm and 100ppm SHAM concentrations using 't.test' function in R (version 4.2.2). A significant difference in the percent mycelial growth inhibition was observed for isolates 16-OP-SB-DIA-035 (P<0.0001),16-OP-SB-DIA-041 (P<0.0005),17-OP-DIA-SOY-029 (P= 0.0009), 14-PHO-SD-19 (P<0.0001) and 16-OP-SB-DIA-064 (P=0.001) comparing 20 ppm and 100 ppm SHAM concentrations. However, for 14-OP-PHO-SD-29 (P=0.07), no significant difference in percent mycelial growth inhibition was observed between 20ppm and 100 ppm SHAM concentrations. When percent mycelial growth inhibition was plotted against respective SHAM concentrations for each isolate (Figure 2.1), a significant increase in the percent mycelial growth was observed for all isolates except non-significant increase for 14-OP-PHO-SD-29. Most of the selected isolates grew well at concentration of 20 ppm SHAM, however a significant inhibition on the mycelial growth has occurred at a concentration of 100 ppm except for the *D. caulivora* isolate 14-OP-PHO-SD-29. Thus, we adopted 20ppm SHAM in our fungicide sensitivity experiments.

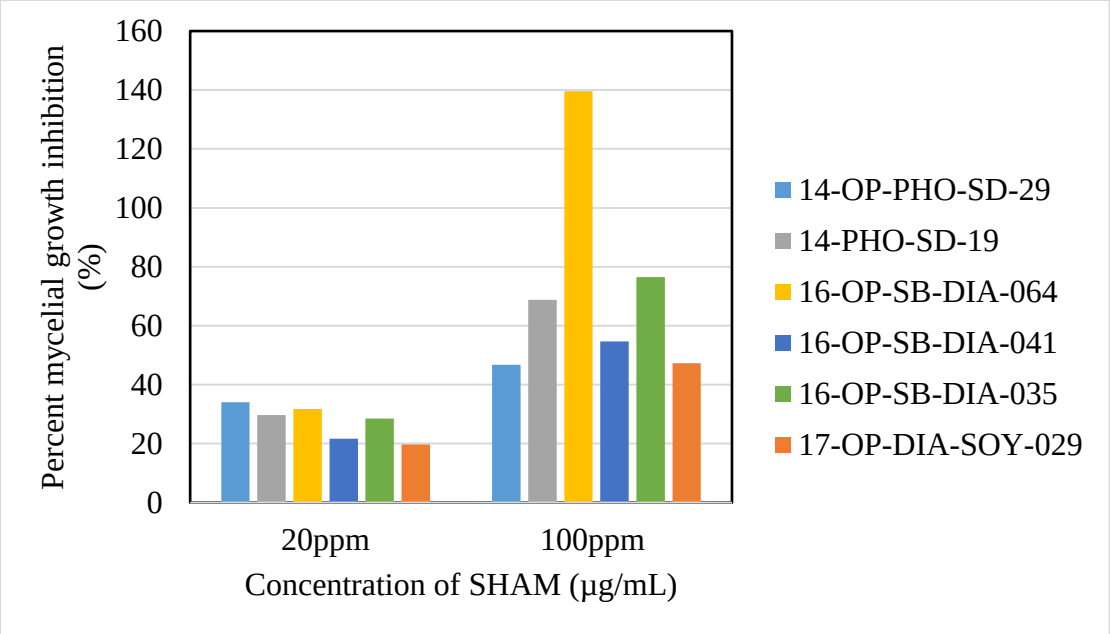

Figure 2.1: Mycelial growth inhibitions of *D.aspalathi* (*n*=2), *D. caulivora* (*n*=2) and *D. longicolla* (*n*=2) on 20ppm and 100ppm concentrations of salicylhydroxamic acid (SHAM) on water agar media.

In order to determine the control treatment for the experiment, four treatments were tested to find out the EC_{50} of selected isolates from *Diaporthe aspalathi* (16-OP-SB-DIA-035 and 16-OP-SB-DIA-041), *D. caulivora* (14-OP-PHO-SD-29 and 17-OP-DIA-SOY-97), and *D. longicolla* (14-PHO-SD-19 and 16-OP-SB-DIA-064). This includes (1) media mixed with acetone-only treatment (water agar+ 1µL/mL acetone) and (2) media mixed with acetone and 20 µg/mL SHAM treatment (water agar+1 µL/mL acetone+20 µg/mL SHAM) treatment, (3) media mixed with 20 µg/mL SHAM treatment (water agar+20 µg/mL SHAM) and (4) media with no acetone and no SHAM treatment (water agar only). The EC_{50} of isolates was calculated considering each treatment as control using above method and compared using ANOVA statistics using 'aov' function in R software. Since no significant difference in the EC_{50} (P>0.05) was observed when treatment (1), (2), (3) and (4) was considered as control treatments when ANOVA was performed (Table 2.1), we used media mixed with acetone only as a control for this experiment.

Table 2.1: Pairwise comparison of the effective concentration of azoxystrobin with acetone, with acetone + SHAM, with SHAM and with no SHAM- no acetone treatment as control to inhibit the mycelial growth by half (EC_{50}) of *D. aspalathi* (*n*=2), *D. caulivora* (*n*=2) and *D. longicolla* (*n*=2) isolates.

Isolates	EC_{50} (µg/mL) [a]				*P*- value[b] (α=0.05)
	With acetone control	With acetone+ SHAM control	With SHAM control	With no SHAM and no acetone control	
D. aspalathi					
16-OP-SB-DIA-35	1.665	1.665	9.821	0.970	0.309
16-OP-SB-DIA-041	5.283	5.283	20.000	3.523	0.256
D. caulivora					
14-OP-PHO-SD-29	0.811	3.367	3.323	0.811	0.655
17-OP-DIA-SOY-97	1.492	1.854	3.030	1.151	0.813
D. longicolla					
14-PHO-SD-19	0.048	0.048	0.049	0.053	1.000
16-OP-SB-DIA-064	0.052	0.053	0.052	0.048	1.000

[a]EC_{50} = Effective concentration of azoxystrobin with acetone, with acetone + SHAM, with SHAM and with no SHAM- no acetone control for inhibiting the growth of *D. caulivora* and *D. longicolla* isolates at 5dpi for *D. caulivora* and *D. longicolla* and 8 dpi for *D. aspalathi*.

[b]ANOVA statistics was performed to compare the EC_{50} obtained using different concentrations and *P* value was determined.

The experiment was designed in a completely randomized design with four plates as replications per SHAM concentration-isolate or fungicide concentration-isolate combination and was performed twice. The mycelial growth measurement of each replication was subjected to test for the normality of the distribution and homogeneity of the experimental repeats by Shapiro-Wilk test and Levene's homogeneity of variance test (Gastwirth et al. 2009), respectively. Results from these tests indicated that mycelial growth data of *D. aspalathi* (*P*<0.001), *D. caulivora* (*P*<0.0014) and *D. longicolla* (*P*<0.002) isolates were not normal and homogeneity of variance (*P*=0.1 for *D. aspalathi*, *P*=0.6 for *D. caulivora* and *P*= 0.9 for *D. longicolla*) was satisfied for the experiments of each species tested.

Since the EC_{50} data was not found to be normal, the EC_{50} values were subjected to non-parametric statistics using 'nparLD' package (Noguchi et al. 2012).

Results

Selection and identification of isolates

A total of 75 isolates belonged to *D. aspalathi* (*n*=15), *D. caulivora* (*n*=19) and *D. longicolla* (*n*=41), and their identity was confirmed using qPCR with the species-specific primer-probe pairs (Table 2.2).

Table 2.2: Species, year of collection, region of origin, and the EC_{50} values of isolates obtained from the *invitro* assay of fungicide sensitivity to azoxystrobin.

Species	Year of collection	Isolate ID	Region	EC_{50}[a] (µg/mL)	Relative Treatment Effects (RTE) based on 95% Confidence Interval [b]
D. aspalathi	2016	16-OP-SB-DIA-001	KY	3.366	0.688 (0.529,0.808)
	2016	16-OP-SB-DIA-006	KY	0.613	0.491 (0.361,0.630)
	2016	16-OP-SB-DIA-012	KY	1.264	0.570 (0.400,0.724)
	2016	16-OP-SB-DIA-027	KY	1.209	0.603 (0.345,0.808)
	2016	16-OP-SB-DIA-030	KY	0.147	0.230 (0.1570.328)
	2016	16-OP-SB-DIA-032	KY	2.057	0.705 (0.645,0.758)
	2016	16-OP-SB-DIA-034	KY	0.247	0.281 (0.162, 0.453)
	2016	16-OP-SB-DIA-035	KY	2.981	0.601 (0.446, 0.736)
	2016	16-OP-SB-DIA-036	KY	2.488	0.590 (0.360,0.781)
	2016	16-OP-SB-DIA-041	KY	4.540	0.629 (0.425,0.791)
	2017	17-OP-DIA-SOY-047	GA	1.320	0.512 (0.363, 0.660)
	2017	17-OP-DIA-SOY-060	KY	0.030	0.138 (0.087,0.229)
	2017	17-OP-DIA-SOY-061	KY	4.291	0.600 (0.411,0.760)
	2017	17-OP-DIA-SOY-074	MO	1.399	0.497 (0.322, 0.674)

Table 2.2: Species, year of collection, region of origin, and the EC_{50} values of isolates obtained from the invitro assay of fungicide sensitivity to azoxystrobin (continued).

Species	Year of collection	Isolate ID	Region	EC_{50}[a] (µg/mL)	Relative Treatment Effects (RTE) based on 95% Confidence Interval [b]
D. caulivora	2014	14-OP-PHO-MI-9	MI	0.037	0.276 (0.146, 0.465)
	2014	14-OP-PHO-SD-23	SD	1.072	0.500 (0.344,0.650)
	2014	14-OP-PHO-SD-29	SD	2.374	0.75 (0.587,0.856)
	2017	17-OP-DIA-SOY-028	MI	0.255	0.271 (0.235,0.310)
	2017	17-OP-DIA-SOY-029	MI	0.815	0.408 (0.230,0.620)
	2017	17-OP-DIA-SOY-033	TN	0.019	0.524 (0.342, 0.699)
	2017	17-OP-DIA-SOY-064	NY	2.538	0.321 (0.160,0.557)
	2017	17-OP-DIA-SOY-084	DE	2.597	0.571 (0.308,0.794)
	2017	17-OP-DIA-SOY-085	WI	1.971	0.336 (0.236, 0.456)
	2017	17-OP-DIA-SOY-087	WI	0.071	0.142 (0.251,0.724)
	2017	17-OP-DIA-SOY-088	WI	0.010	0.850 (0.776,0.890)
	2017	17-OP-DIA-SOY-090	WI	0.149	0.631(0.450, 0.777)
	2017	17-OP-DIA-SOY-091	WI	1.823	0.562 (0.365,0.740)
	2017	17-OP-DIA-SOY-097	WI	1.887	0.533(0.356, 0.700)
	2017	17-OP-DIA-SOY-098	WI	0.038	0.331(0.253, 0.428)
	2017	17-OP-DIA-SOY-099	WI	0.383	0.822 (0.724, 0.868)
	2022	22-OP-DIA-SOY- 03	MN	0.631	0.429 (0.307,0.561)
	2022	22-OP-DIA-SOY- 04	MN	0.055	0.598 (0.441,0.737)
	2022	22-OP-DIA-SOY- 18	SD	0.753	0.700 (0.580, 0.786)
D.longicolla	2014	14-OP-PHO-IA-1	IA	1.027	0.595 (0.411,0.755)
	2014	14-OP-PHO-IA-2	IA	2.183	0.588 (0.339,0.797)
	2014	14-OP-PHO-IA-4	IA	0.045	0.374 (0.293,0.464)
	2014	14-OP-PHO-IL-13	IL	0.061	0.438 (0.352,0.529)
	2014	14-OP-PHO-IN-8	IN	0.938	0.373 (0.208, 0.577)
	2014	14-OP-PHO-MI-12	MI	2.035	0.368 (0.159, 0.648)
	2014	14-OP-PHO-SD-16	SD	8.456	0.881(0.748,0.944)
	2014	14-OP-PHO-SD-17	SD	0.057	0.284 (0.161, 0.455)

Table 2.2: Species, year of collection, region of origin, and the EC_{50} values of isolates obtained from the invitro assay of fungicide sensitivity to azoxystrobin (continued).

Species	Year of collection	Isolate ID	Region	EC_{50}[a] (µg/mL)	Relative Treatment Effects (RTE) based on 95% Confidence Interval [b]
D. longicolla	2014	14-OP-PHO-SD-19	SD	0.216	0.373 (0.256,0.507)
	2014	14-OP-PHO-SD-20	SD	1.098	0.520 (0.307,0.727)
	2014	14-OP-PHO-SD-31	SD	0.018	0.279 (0.208, 0.366)
	2016	16-OP-SB-DIA-025	KY	0.939	0.457 (0.241, 0.692)
	2016	16-OP-SB-DIA-037	KY	0.030	0.249 (0.149, 0.389)
	2016	16-OP-SB-DIA-052	IA	0.057	0.215 (0.129,0.360)
	2016	16-OP-SB-DIA-058	IN	0.800	0.486 (0.292, 0.684)
	2016	16-OP-SB-DIA-064	IN	0.646	0.504 (0.339, 0.667)
	2016	16-OP-SB-DIA-065	IN	0.045	0.253 (0.122, 0.460)
	2016	16-OP-SB-DIA-070	IN	1.460	0.588 (0.465,0.701)
	2016	16-OP-SB-DIA-072	IN	0.64	0.653 (0.468, 0.800)
	2016	16-OP-SB-DIA-077	IN	0.104	0.531 (0.459, 0.602)
	2016	16-OP-SB-DIA-078	IN	0.140	0.423 (0.236, 0.636)
	2016	16-OP-SB-DIA-081	IN	2.776	0.321 (0.270, 0.345)
	2016	16-OP-SB-DIA-083	IN	1.296	0.677 (0.507,0.809)
	2017	17-OP-DIA-SOY-003	MS	0.50	0.676 (0.623, 0.724)
	2017	17-OP-DIA-SOY-004	MS	1.68	0.406 (0.306,0.516)
	2017	17-OP-DIA-SOY-009	MS	0.396	0.506 (0.322, 0.688)
	2017	17-OP-DIA-SOY-022	SD	0.047	0.373 (0.257, 0.506)
	2017	17-OP-DIA-SOY-031	MI	0.125	0.247 (0.119, 0.449)
	2017	17-OP-DIA-SOY-037	SD	2.052	0.690 (0.486,0.837)
	2017	17-OP-DIA-SOY-054	ND	0.293	0.300 (0.138,0.541)
	2017	17-OP-DIA-SOY-055	SD	3.92	0.704 (0.485, 0.855)
	2017	17-OP-DIA-SOY-058	KY	1.640	0.516 (0.304,0.722)
	2017	17-OP-DIA-SOY-068	MO	0.028	0.408 (0.244, 0.596)
	2017	17-OP-DIA-SOY-083	DE	0.323	0.564 (0.371,0.738)
	2017	17-OP-DIA-SOY-102	WI	0.004	0.343(0.215, 0.505)
	2017	17-OP-DIA-SOY-127	MS	1.125	0.672 (0.465,0.826)

Table 2.2: Species, year of collection, region of origin, and the EC_{50} values of isolates obtained from the *invitro* assay of fungicide sensitivity to azoxystrobin (continued).

Species	Year of collection	Isolate ID	Region	EC50[a] (µg/mL)	Relative Treatment Effects (RTE) based on 95% Confidence Interval [b]
D. longicolla	2017	17-OP-DIA-SOY-128	AL	2.092	0.728 (0.532,0.861)
	2017	17-OP-DIA-SOY-129	WI	3.545	0.678 (0.465,0.889)
	2018	18-OP-DIA-SOY-16	SD	0.028	0.354 (0.302,0.409)
	2022	22-OP-DIA-SOY- 17	SD	0.021	0.187(0.124, 0.360)
	2022	22-OP-DIA-SOY- 19	SD	0.818	0.419 (0.222,0.647)

[a]EC_{50} = Effective concentration of azoxystrobin for inhibiting the growth of *D.aspalathi* at 8dpi and *D. caulivora* and *D. longicolla* isolates at 5 dpi.

[b]EC_{50} was analyzed using nonparametric statistics (Shah and Madden 2004) and expressed as relative treatment effect. Test statistics were calculated using nparLD package (Noguchi et al. 2012). 95% confidence intervals in parentheses.

***In vitro* sensitivity assay**

The ANOVA-type statistics showed a significant effect (P=0.002) of EC_{50} on the isolates (expressed as relative treatment effect (RTE)). The EC_{50} of *D. aspalathi* ranged from 0.030 µg/mL to 4.540 µg/mL (mean EC_{50}=1.595 µg/mL) and significant differences in EC_{50} were observed among isolates. Isolate 16-OP-SB-DIA-032 from Kentucky had the highest RTE value of 0.705 with 95% confidence intervals from 0.645 to 0.758, while isolate 17-OP-DIA-SOY-060 showed a lower RTE value of 0.138 and 95% confidence interval from 0.087 to 0.229. When the frequency of isolates was plotted against respective EC_{50} value range (class interval of 1 µg/mL), a rightly skewed unimodal distribution was obtained and EC_{50} of 5 isolates was in the range of 0.0 to 1.0 µg/mL, 4 isolates in 1.1 to 2.0 µg/mL, 3 isolates in 2.1 to 3.0 µg/mL, 1 isolate in 3.1 to 4.0 µg/mL and 2 isolates in 4.1 to 5.0 µg/mL (Figure 2.2).

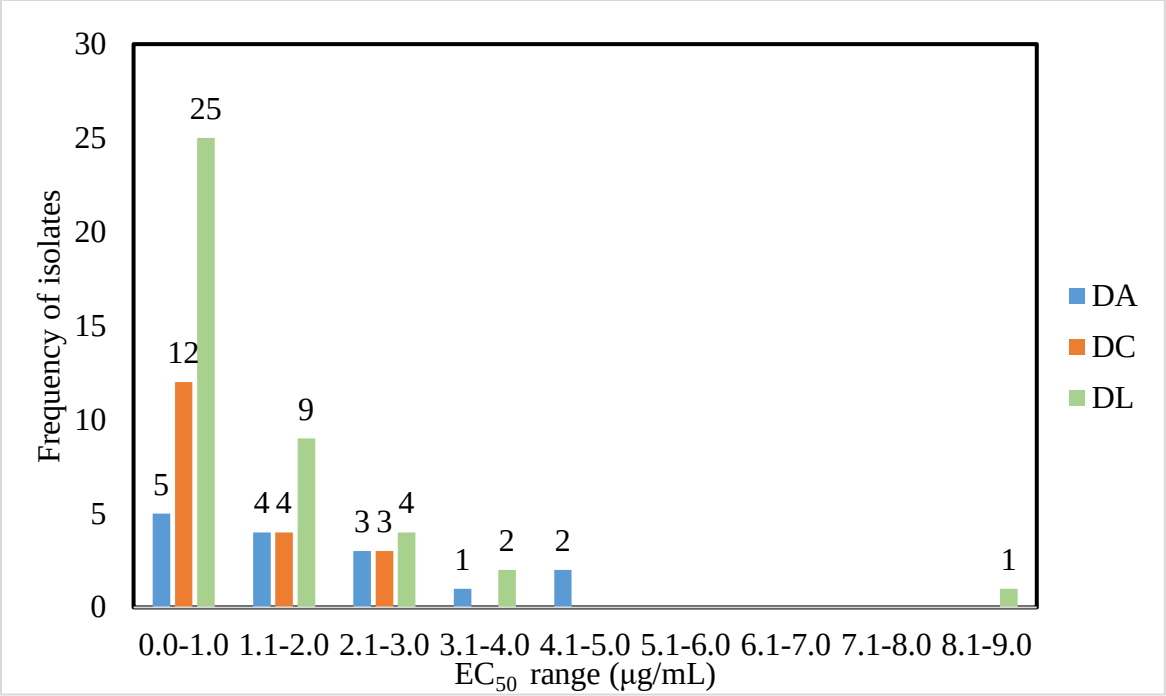

Figure 2.2: Frequency distribution of effective concentration of azoxystrobin at which the mycelial growth was inhibited by 50% (EC_{50} (µg/mL)) for 15 isolates of *D. aspalathi*, 19 of *D. caulivora* and 41 of *D. longicolla* collected from different locations between 2014 and 2017. Mean EC_{50} across all replications of each isolate (µg/mL) on x-axis and number of isolates on y-axis. Individual isolates are grouped in class intervals of 1 mg/L.

For *D. caulivora*, ANOVA-type statistics showed significant differences (*P*=0.014) among EC_{50} values of the isolates (expressed as RTE) of *D. caulivora* across different years from different states. The range of EC_{50} of *D. caulivora* was 0.010 µg/mL to 2.597 µg/mL (mean EC_{50}= 0.968 µg/mL). No significant difference in EC_{50} among isolates of *D. caulivora* was observed. When the frequency of isolates was plotted against respective EC_{50} value range (class interval of 1 µg/mL), a rightly skewed unimodal distribution was obtained with EC_{50} of 12 isolates in the range of 0.0 to 1.0 µg/mL, four isolates in 1.1 to 2.0 µg/mL and three isolates in 2.1 to 3.0 µg/mL (Figure 2.2).

For *D. longicolla*, ANOVA-type statistics showed a significant effect of EC_{50} (*P*=0.003) on the isolates. The EC_{50} of the isolates ranged from 0.004 µg/mL to 8.456 µg/mL with mean EC_{50} of 1.096 µg/mL (Table 2.2). No significant differences in EC_{50} were observed among

isolates of *D. longicolla*. When the frequency of isolates was plotted against respective EC_{50} value range (class interval of 1 µg/mL), a rightly skewed unimodal distribution was obtained with EC_{50} of 25 isolates in the range of 0.0 to 1.0 µg/mL, nine isolates in the range of 1.1 to 2.0 µg/mL, four isolates in the range of 2.1 and 3.0 µg/mL, two isolates in 3.1 to 4.0 µg/mL and one isolate in 8.1 to 9 µg/mL (Figure 2.2). The isolate with the highest EC_{50} (8.45 µg/mL) was from Union County, South Dakota.

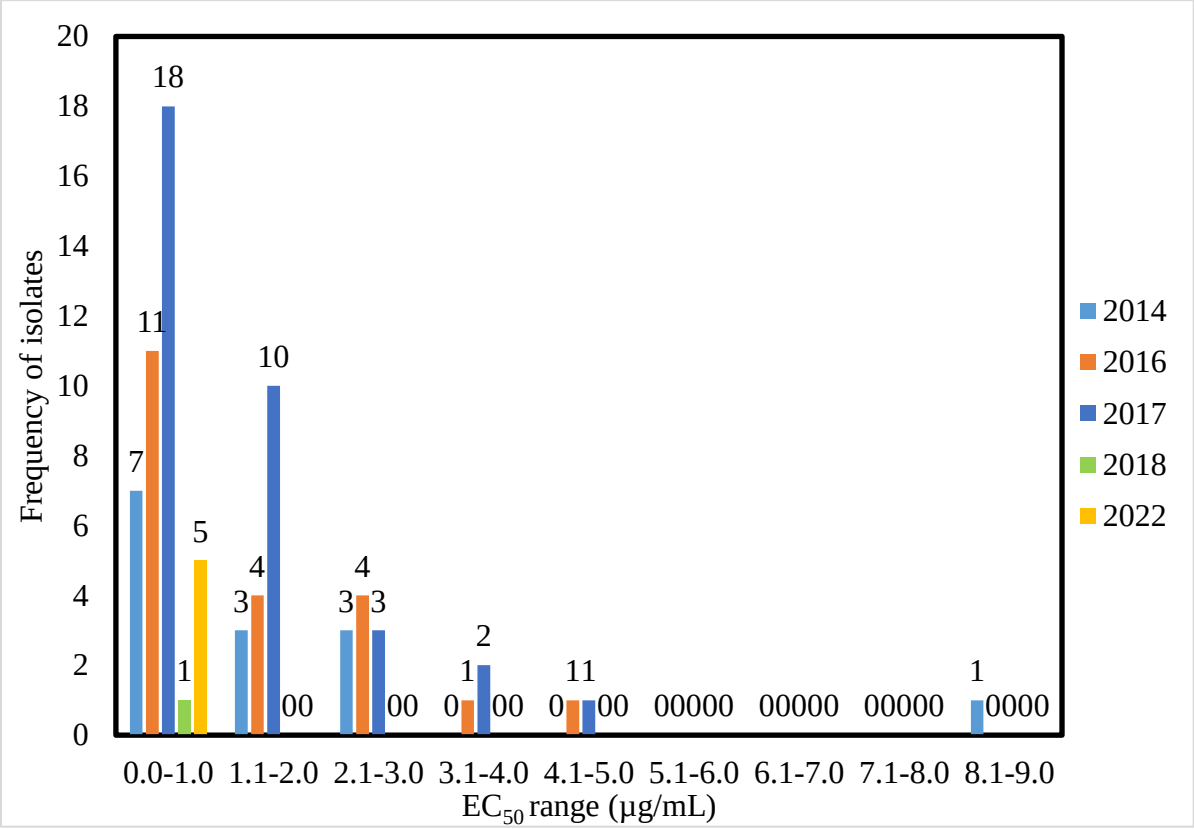

Figure 2.3: Frequency distribution of the effective concentration of azoxystrobin required to inhibit mycelial growth by 50% (EC_{50}) in 75 isolates of *D. aspalathi*, *D. caulivora* and *D. longicolla* collected from 2014 to 2022 from sixteen U.S states. Values were determined based on conventional growth plate assays. Individual isolates are grouped in class intervals of 1 mg/L.

Frequency distribution of EC_{50} of 75 isolates of *D. aspalathi*, *D. caulivora* and *D. longicolla* grouped to their year of collections from 2014 to 2022 indicated a greater number of

isolates in the low EC_{50} range, suggesting most isolates of *Diaporthe* screened are sensitive to azoxystrobin (Figure 2.3).

Discussion

This study represents a broad investigation into the sensitivity of the *Diaporthe* spp. to fungicides in soybeans in the United States. QoI fungicides carry a high risk of developing fungicide resistance (FRAC 2023) due to their single site and mode of action. A QoI fungicide, 'Quadris®'with, azoxystrobin as the active ingredient, is labeled for protection against pod and stem blight caused by *D. sojae* Lehman and *D. longicolla*. For this reason, we selected azoxystrobin to determine the fungicide sensitivity to *Diaporthe* spp. If QoIs hinder normal respiration, alternative oxidase enzymes in fungi may facilitate an alternative respiratory pathway (Kaneko and Ishii 2009). Although QoI fungicides inhibit spore germination and early fungal growth, we opted to conduct mycelial growth assays to monitor fungicide resistance. This is because the in-vitro production of ascospores, which is considered the primary source of inoculum, is highly challenging and time-consuming (Hosseini et al. 2020, Santos and Phillips 2009). Furthermore, mycelial growth assays were chosen due to their ease of experimentation and because they have been used to assess the fungicide sensitivities of *D. caulivora* and *D. longicolla* (Floyd and Malvick 2022).

In this study, a total of 75 isolates were examined to determine their susceptibility to the QoI fungicide, azoxystrobin. A shift in fungicide sensitivity of isolates is determined by comparing exposed individuals to a baseline isolate. However, in this study, we were unable to include a baseline isolate for any of the species as they were not available. Consequently, there were no baseline isolates against which the EC_{50} of *Diaporthe* isolates could be compared. However, the EC_{50} range of *D. caulivora* in our study falls within the EC50 range of *D. helianthi*

Muntañola-Cvetković et al. (0.004 to 4.027 µg/mL) in sunflower to azoxystrobin (Mohan et al.202X. unpublished). Another study conducted by Mondal et al. (2007) reported an EC_{50} range of 0.03 to 0.45 µg/mL for *D. citri* Wolf in *Citrus* spp. to azoxystrobin which falls within the EC_{50} range of *D. aspalathi*, *D. caulivora* and *D. longicolla* in our studies. A unimodal rightly skewed graph was also obtained when the frequency of isolates grouped each into their year of the collection was plotted against classes of EC_{50} of 1 µg/mL (Figure 2.3). However, there was no trend observed in the year of collection when the frequency of isolates was plotted against their EC_{50} groups based on their year of collection. In addition, the sample size was low in each species such as *D. aspalathi* (*n*=15), *D. caulivora* (*n*=19) and *D. longicolla* (*n*=41), thus we cannot confirm a shift in sensitivity among each species to azoxystrobin. However, currently, with the isolates tested in this study, molecular detection of common mutation responsible for QoI resistance such as G143A mutation is performed with amplification of cyt *b* using Polymerase Chain Reaction (PCR) with primers specific to *D. aspalathi*, *D. caulivora* and *D. longicolla* species in soybean. The sequences are analyzed for the codon at 143rd region of cyt *b* gene comparing with available *cyt b* gene sequence of *D.longicolla* in NCBI database.

In our study, initially, we preferred to include isolates from multiple U.S states for the inclusion of geographical variability in isolates, after which we selected randomly from the available collection. There are other techniques by which potential fungicide-resistant isolates can be sampled. For example, Lowder et al. (2023) compared the efficiency of different methods, such as worn gloves of workers vs visual detection and collection of fungal isolates with cotton swabs from leaves vs using rotating arm impaction spore traps to sample the QoI fungicide-resistant isolates of *Erysiphe necator* (Schweinitz) Burrill in commercial vineyards in Oregon, Washington, and California states. They used swabs collected from worn gloves of workers as a

method of sampling vs visual detection and collection of fungal isolates with cotton swabs from leaves vs using rotating arm impaction spore traps and comparison was performed. They identified swabs collected from worn gloves of workers as an efficient and economic method of sampling *E. necator* isolates from vineyards.

Even though the influence of geographical variability in the sensitivity among *Diaporthe* spp. in soybean to azoxystrobin can be studied with the isolates from different regions in the U.S, we did not have enough isolates from each U.S state. The adoption of conidial germination test instead of mycelial growth inhibition assay would have changed the EC_{50} values of the isolates of *D. aspalathi, D. caulivora* and *D. longicolla* (Chen et al. 2015, Sautua and Carmona 2021) primarily due to the difference in exposure time of fungicide concentrations to conidial germination and mycelial growth assays, the difference in stages of the fungal growth (spore vs mycelia), and difference in how the effect of fungicide concentrations on fungus measured (conidial germination vs diametric growth of mycelia). *Invitro* assays can be considered as a cornerstone in the detection of fungicide sensitivity of fungal population, however, molecular diagnostic assays are less resource intensive and can reliably and timely detect single nucleotide polymorphisms (SNPs) and so require that point mutations causing resistance are known. These methods are particularly useful when the resistance mechanism is linked to specific genetic mutations or changes. Detection involves Polymerase Chain Reaction (PCR) assays based on hybridization or amplification with allele-specific probes or primers, use of restriction enzymes or sequencing. For example, for identifying QoI fungicide-resistant mutants in *Cercospora sojina* Hara population causing frogeye leaf spot disease in soybean, Standish et al. (2015) used PCR amplification of the cyt *b* gene region of isolates sampled from the fields of Mississippi and

undergone PCR-restriction fragment length polymorphism (RFLP) to detect potential mutation at codon 143 of the gene.

While *in-vitro* assays are only preliminary and cannot provide a definitive picture of fungicide sensitivity in the fungal population, the information obtained can be useful for planning future investigations into fungicide resistance screening studies and monitoring any changes in the fungal population's response to fungicides. *In-planta* experiments, conducted in both greenhouse and field conditions, can help determine the reaction of isolates to multiple QoI fungicides and provide a more conclusive understanding of this issue. Even though azoxystrobin was found to be effective against *Diaporthe* in soybean in this study, sole dependence on QoI fungicides would increase the risk of fungicide resistance. Therefore, integrated disease management practices such as crop rotation with non-host crops, adoption of *Diaporthe* resistant cultivars against, and need-based fungicide application and rotations based on weather data (for example, a new smartphone application, Sporecaster, (Willbur et al. 2018a, 2018b)) would be helpful in effectively managing diseases associated with *Diaporthe*.

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CHAPTER THREE: DETERMINATION OF DISTINCT FORMS OF RESISTANCE TRIGGERED BY SEED AND STEM INFECTION OF *DIAPORTHE LONGICOLLA* IN SOYBEAN (*GLYCINE MAX* L.)[2]

Abstract

Species of *Diaporthe* can cause diseases in soybeans (*Glycine max* L.) and produced an estimated yield loss of 0.2 million metric tons in 2022. While *D. longicolla* can cause Diaporthe seed decay and pod and stem blight, little is known whether the genetic mechanism underlying resistance to this pathogen is the same. The objective of this study was to identify the different forms of resistance to *D. longicolla* as a stem and seed pathogen, using a total of 39 genotypes. The experimental design was completely randomized design with two experiments and used a North Dakota isolate of *D. longicolla*. To inoculate stems, a mycelial plug was pressed against a wound created on the plants during their second trifoliate growth stage. The plants were then incubated at 24±3°C with 90% humidity for 7 days. For seed infection, surface sterilized seeds were immersed in mycelial suspension for four hours. The plates were incubated at 23±2°C under diffused light for 7 days. A significant effect of genotype on disease rating was observed for both stem (P<0.0004) and seed (P<0.0003) infections. For stem and seed infections, twenty-nine and six accessions respectively, showed lower disease severity compared to their respective susceptible checks 'Hawkeye' and 'PI 37161'. However, five genotypes showed less susceptibility to both stem and seed infection. A non-significant correlation (r=0.15, P=0.3) was found between the severity of stem infection and seed decay. The results indicate that there are

[2] A paper to be submitted to the journal *Plant Health Progress* published by the American Phytopathological Society

possibilities of different resistance mechanisms for seed and stem infection caused by *D. longicolla,* which needs further investigation.

Introduction

Species of *Diaporthe* Nitschke [syn. *Phomopsis* (Sacc.) Bubák] have been identified as endophytes, saprophytes, and latent pathogens in several crops, including soybean (*Glycine max* L.) (Lehman 1923; Zhao et al. 2022a, Li and Chen 2013). Among diseases associated with *Diaporthe* in soybeans, Diaporthe (Phomopsis) seed decay, and pod and stem blight were listed among the top soybean stem diseases in the United States (29 states) and Canada (Ontario) in 2022 (Allen et al. 2023).

Diaporthe seed decay can infect soybeans at any growth stage, but the seeds are more vulnerable during the pod filling (R5) to full maturity (R8) growth stages (Fehr et al.1971). The primary cause of the disease is *Diaporthe longicolla* (Hobbs) Santos et al (syn. *Phomopsis longicolla* Hobbs), but other species of *Diaporthe* may be involved as a pathogen complex (Petrovic et al. 2021, Hosseini et al. 2020). For example, species of *Diaporthe* such as *D. aspalathi* van Rensburg et al., *D. bacilloides* Petrović et al., *D. caulivora* (Athow and Cadwell) Santos et al., *D. flavescens* Petrović et al., *D. insulistroma* Petrović et al., *D. kongii* Shivas et al., *D. sojae* Lehman, *D. ueckaere* Udayanga & Castl. and *D. unshiuensis* Huang et al. were recovered from soybean seeds collected from 17 locations in eight U.S. states (Delaware, Iowa, Louisiana, Michigan, Mississippi, South Dakota, Tennessee, and Wisconsin) and observed to develop seed decay and/or seedling necrosis (Petrovic et al. 2021). In addition to causing seed decay, the species complex of *Diaporthe* can compromise seed/grain quality, germination, and stand establishment (Li et al. 2011, Sinclair 1993). In 2022, Diaporthe seed decay caused an

estimated total yield loss of 0.1 million metric tonnes across 29 states in the U.S. (Allen et al. 2023).

Pod and stem blight, caused by *D. sojae* and *D. longicolla* in the U.S., is mostly observed from the beginning full-stage (R6) through the beginning maturity (R8) growth stages (Anonymous 2019b). Symptoms include black raised specks arranged in linear rows along the mature soybean stem and pods, which are asexual reproductive structures of the fungus, which are called pycnidia. In 2022, the diseases caused a total yield loss of 0.1 million metric tons across 29 U.S. states (Allen et al. 2023).

For all *Diaporthe* diseases, the causal fungi survive the winter as dormant mycelia (Grijalba and Ridao 2012) in the soil, infected crop residue, and weeds like velvetleaf (*Abutilon theophrasti*). When there is widespread rain occurs during the early growth stages of the crop, both ascospores and /or conidia from perithecia and pycnidia, respectively (Backman et al.1985, Padgett 1992, Xue et al.2007) are water splashed onto the leaves of the plant. Symptoms of the diseases start to appear when warm and humid weather occurs during the late reproductive growth stages of the crop, and they can extend until harvest (Anonymous 2019b).

Management of *Diaporthe*-associated diseases involves cultural practices, foliar fungicide application, use of seed treatments, weed management, and host resistance (Anonymous 2019a, b). Cultural practices like conventional tillage and crop rotation with non-host crops decrease the fungal colonization in the field and the spread of spores (Anonymous 2019a, b, Li et al. 2015). Harvesting mature pods on time can reduce disease incidence and severity, but it may not be possible if there are environmental conditions inhibiting timely harvest such as late-season rain/snow. *Diaporthe*-associated diseases can limit yield by 1.6t/ha if the harvest is delayed due to late-season rains (Allen et al. 2019). For example, in 2018, there was a 1600% increase in

seed/grain diseases in soybeans compared to previous years, primarily due to Diaporthe seed decay caused by excessive rainfall during late reproductive growth stages and delayed harvest (Bradley et al. 2021). Foliar fungicides can be used as an option, but recent studies have raised concerns about the effectiveness of fungicide applications. Batzer and Mueller (2020) found a higher prevalence of *Diaporthe* in soybean stems, leaves, and seeds from a mix of fluxapyroxad and pyraclostrobin treated plots compared to untreated control plots. A study by Cross et al. (2012) showed no significant difference in the percentage of infected harvested seeds and the severity of pod and stem blight between plots treated with azoxystrobin [Quinone outside inhibitor fungicide (QoI) belonging to Fungicide Resistance Action Committee (FRAC) 11 class of fungicides] and control plots. Therefore, host resistance can be considered as an economically and environmentally viable solution for managing *Diaporthe*-associated diseases.

In the past decade, field screening of soybean accessions from the United States Department of Agriculture (USDA) Soybean Germplasm for resistance to *D. longicolla*, the causal agent of Diaporthe seed decay has been conducted (Li et al. 2010b, Li 2011, Li et al. 2017, Li et al. 2023). Over five years, approximately 135 genotypes from 28 different geographic regions were field tested in Arkansas, Mississippi, and Missouri to assess their reaction to the causal pathogens of Diaporthe seed decay in 2009, 2010, 2011, and 2012 (Li et al. 2015). Both non-inoculated as well as inoculated treatments were included in the field trials. For inoculated treatment, spore suspension made from 30 to 45 days old cultures of *D. longicolla* were used and after the harvest of the crop, 30 to 50 random seeds from each plot in each trial were plated on potato dextrose agar (PDA) plate with a pH 4.8. The number of seeds infected with *D. longicolla* was recorded and calculated as percent seed infection. From this testing, fifteen accessions were found to be resistant (Li et al. 2015).

In addition to seed infection, investigations into the reaction of soybean genotypes to stem infection by *D. longicolla* were also performed (Ghimire et al. 2019, Mohan et al. 2023, Kontz et al. 2016). Multiple inoculation methods such as stem wound method, toothpick method, mycelial contact method, and spore injection method were evaluated by Ghimire et al. (2019) using multiple isolates of *D. aspalathi, D. caulivora* and *D. longicolla* in 'Bragg' (for *D. aspalathi*) and 'Hawkeye' (for *D. caulivora* and *D. longicolla*) and determined stem wound and toothpick inoculation techniques as effective in causing infection in greenhouse conditions. Kontz et al. (2016) screened nine PI lines for their reaction to stem infection by an isolate of *D. longicolla* (DP01-422). They found 'PI 612708C', 'PI 417507', and 'PI 507705' were resistant when compared to susceptible check 'Williams 82'. Mohan et al. (2023) screened the aggressiveness of multiple isolates of *D. longicolla* in 'PI 612708 C', 'PI 417507', 'PI 5077705' including 'Hawkeye' as susceptible check and identified PI 417507 as a resistant genotype. However, PI 612708C, PI 417507, and PI 507705 observed to be resistant were not tested under field conditions by Mohan et al. (2023) or Kontz et al. (2016). Although efforts are currently ongoing to evaluate the resistance of soybean varieties to *D. longicolla* , there is limited information available regarding the reaction of soybean genotypes to *D. longicolla* as the causal pathogen of pod and stem blight.

The purpose of this study was to identify the different types of resistance found in soybean accessions that show resistance to *D. longicolla* in either field or greenhouse conditions. Specifically, we aimed to assess 39 soybean accessions that were previously identified as resistant to *D. longicolla* as a stem or seed pathogen in field trials or greenhouse experiments. We conducted seed and stem infection experiments of these accessions in the laboratory and/or

greenhouse. The findings from this study will provide soybean breeders with valuable information on various forms of resistance to *D. longicolla*, which can be used in future breeding efforts.

Materials and methods

One isolate of *D. longicolla* (17-OP-DIA-SOY-054) was used in this study to screen for both stem and seed resistance of selected soybean germplasm. The isolate was recovered from infected diseased soybean stems from Barnes County in North Dakota in 2017. The sampled plants exhibited pod and stem blight symptoms, such as pycnidia on the stem. The fungus was isolated from cut stem pieces (1 cm long) after undergoing surface sterilization. The sterilization process involved treating the stem pieces with 0.05% sodium hypochlorite for 1 min, followed by 70% ethanol for 1 min. The pieces were then washed twice with sterile water and air-dried on filter paper in a laminar airflow hood.

Three stem pieces from each plant were plated on full-strength Potato Dextrose Agar (PDA) media supplemented with 0.3g/L of streptomycin sulfate to avoid bacterial contamination. The plates were then incubated at 23±2ºC for 10 days under 12 hours of alternating light and dark conditions. Isolates that emerged from the plant parts were recovered by hyphal tipping from the leading colony edge and transferred to fresh PDA plates. These fresh plates were incubated under the same conditions as described previously. The cultures were observed under a microscope to identify colony growth, pycnidia development, and conidia formation characteristic of *D. longicolla* in soybeans (Petrovic et al. 2021, Udayanga et al. 2015).

For molecular identification, the mycelia from 7-day-old cultures were harvested and ground in a 1.5mL Eppendorf tube with a spatula sterilized with 70% ethanol. The mycelia were ground in 700µL of the lysis buffer, which consisted of 1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, and 10% sodium dodecyl sulfate. The tube was then stored in a heating block at 65ºC for 15

minutes. Afterward, 150 µL of potassium acetate (60 ml of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of distilled water) was added to the tube. The tubes were subjected to centrifugation at 18,000 rpm for 3 min, and the supernatant was transferred to a new 1.5mL Eppendorf tube. To the supernatant, an equal volume of isopropyl alcohol was added. The mixture was centrifuged at 15,000 rpm for 1 min, and the resulting pellet was washed with 500µL 70% ethanol. The pellet was then centrifuged at 10,000 rpm for 1 min and air-dried. To dilute the DNA precipitate, 20µL of Tris-EDTA (10 mM Tris and 1 mM EDTA) was added, and the quality of the DNA was assessed using a Nanodrop spectrophotometer.

The extracted DNA underwent quantitative Polymerase Chain Reaction (qPCR) for confirmation of the identity of *D. longicolla* using the PL-3 primers-probe pair (Kontz et al. 2016). The qPCR assays were performed using the Applied Biosystems QuantStudio 3 and 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). The qPCR mixture consisted of 10 µL of TaqMan® Universal Master Mix II (Applied Biosystems), 0.4µL each of forward and reverse primers, 0.2µL of fluorescent TaqMan® probe, and 7 µL of sterile nuclease-free water for each sample of DNA diluted to 2 ng/µL. The qPCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (Kontz et al. 2016).

Soybean accessions

A total of 39 soybean accessions were screened for stem and seed resistance to *D. longicolla.* They originated from 10 countries (Germany, China, Japan, Pakistan, South Africa, South Korea, Uganda, the United States, Uruguay, and Taiwan) and belonged to maturity groups (MG) 0 to IX (Table 3.1).

Table 3.1: Maturity group, accessions and references from which accessions were used in this study.

Maturity Group	Accession	Reference	Comments
0	PI 417507	Kontz et al. (2016) Mohan et al. (2023)	Found resistant to stem infection by *D. longicolla*
0	'Barnes' (PI 614831)	-	Unknown reaction to *D. longicolla* as a stem and seed pathogen
I	PI 612715	Kontz et al. (2016)	Susceptible to stem infection by *D. longicolla*
I	'Vinton 81' (PI 548625)	Li et al. (2015)	Susceptible to seed infection by *D. longicolla*
I	'Blackhawk' (PI 548516)	Backman et al. (1985)	Resistant to stem infection by *D. caulivora*
I	'10049-142-31'	Included from previous study conducted in nursery by USDA-Agricultural Research Service, MS	Unknown reaction to *D. longicolla* as a stem and seed pathogen
II	PI 603756	Smith et al. (2008)	Low seed infection by *D. longicolla*
II	PI 547453	Gillen et al. (2012)	More seed infection (belonging to isolines of genotype Clark) by *D. longicolla*
II	'Hawkeye 63' (PI 548578)	Backman et al. (19859	Progeny of Hawkeye and Blackhawk which are found to be resistant to stem infection by *D. caulivora*
II	'Hawkeye' (PI 548577) [a]	Mohan et al. (2023)	Susceptible check to stem infection by *D. longicolla*
III	'Williams 82' (PI 518671)	Kontz et al. (2016)	Susceptible check to stem infection by *D. longicolla*
III	PI 398697	Li et al. (2010b)	Low seed infection by *D. longicolla*
III	PI 398752	Li et al. (2010b)	Low seed infection by *D. longicolla*

Table 3.1: Maturity group, accessions and references from which accessions were used in this study (continued).

Maturity Group	Accession	Reference	Comments
III	PI 417361	Li et al. (2010b)	Low seed infection by *D. longicolla*
III	PI 504488	Li et al. (2015)	Low seed infection by *D. longicolla*
III	PI 689003	Smith et al. (2019)	Resistant to *D. aspalathi* stem infection but susceptible to *D. longicolla* seed infection
III	PI 548298	Li et al. (2015)	Susceptible check to seed infection by *D. longicolla*
III	PI 547652	Gillen et al. (20120	More seed infection (belonging to isolines of genotype Clark) by *D. longicolla*
III	'DB06×0006-93'	Included from previous study conducted in nursery by USDA-Agricultural Research Service, MS	Unknown reaction to *D. longicolla* as a stem and seed pathogen
IV	PI 158765	Li et al. (2015)	Low seed infection by *D. longicolla*
IV	PI 235335	Li et al. (2015)	Low seed infection by *D. longicolla*
IV	PI 652443	Pathan et al. (2009)	Resistant to seed infection by *D. longicolla*
IV	PI 642055	Paris et al. (2006)	Resistant to stem infection by *D. aspalathi*
IV	'AP 350' (PI 556625)	Li et al. (2015)	Susceptible check to seed infection by *D. longicolla*
IV	PI 371611[b]	Li et al. (2015)	Susceptible check to seed infection by *D. longicolla*
IV	PI 547432	Gillen et al. (2012)	More seed infection (belonging to isolines of genotype Clark) by *D. longicolla*

Table 3.1: Maturity group, accessions and references from which accessions were used in this study (continued).

Maturity Group	Accession	Reference	Comments
IV	PI 591491	Gillen et al. (2012)	More seed infection (belonging to isolines of genotype Clark) by *D. longicolla*
IV	PI 547610	Gillen et al. (2012)	More seed infection (belonging to isolines of genotype Clark) by *D. longicolla*
IV	PI 591490	Gillen et al. (2012)	More seed infection (belonging to isolines of genotype Clark) by *D. longicolla*
IV	PI 417561	-	Included with other maturity group (MG) IV varieties since screening genotypes in MG IV might be beneficial for breeding programs adapted to the southern U.S as problems caused by *D. longicolla* are more impactful in southern U.S states than in northern states.
V	11043-211-10	Li et al. (2023)	Low seed infection by D. longicolla
V	PI 381668	Li et al. (2015)	Low seed infection by *D. longicolla*
V	PI 407749	Li et al. (2015)	Low seed infection by *D. longicolla*
V	PI 567381 B	Li et al. (2011)	Low seed infection by *D. longicolla*
V	'Bay' (PI 553043)	Backman et al. (1985)	Resistant to stem infection by *D. caulivora*
V	'AP 55' (PI 556626)	Backman et al. (1985)	Susceptible to stem infection by *D. caulivora*

Table 3.1: Maturity group, accessions and references from which accessions were used in this study (continued).

Maturity Group	Accession	Reference	Comments
VI	'Tracy-M' (PI 548984)	Backman et al. (1985)	Resistant to stem infection by *D. caulivora*
VII	'Bragg' (PI 548660)	Keeling (1982)	Moderately susceptible to *D. caulivora*
IX	'Jupiter -R' (PI 548973)	Backman et al. (1985)	Susceptible to stem infection by *D. caulivora*

[a] Susceptible check of stem infection experiment

[b] Susceptible check of seed infection experiment

The genotypes Hawkeye (PI 548577) and PI 371611 were considered susceptible checks for stem (Mohan et al. 2023) and seed experiments (Li et al. 2015), respectively (Table 3.1).

Stem resistance experiment under greenhouse conditions

Three seeds of each accession were planted in a 475 mL plastic cup filled with Promix general purpose growing mix (Premier Tech Growers and Consumers, Quebec, Canada), which contained sphagnum moss (75 to 85%), perlite, limestone, and a wetting agent. Five grams of Multicote 14-14-16+ Micronutrients (Magnesium, Sulfur, Iron, Manganese, Copper, Molybdenum, Zinc and Boron) fertilizer (Haifa Group, Altamonte Springs, Florida, USA) was added per cup before planting. The cups were maintained at a temperature of 24±3ºC under 12 hours of light and dark conditions (Ghimire et al. 2019, Kontz et al. 2016, Mohan et al. 2023) throughout the experiment. The experiment design was completely randomized design (CRD) with eight replications per experiment per accession, and it was performed twice. Each plant was considered a replication, and two plants were kept in each cup for the experiment. The extra plant from each cup was removed before the inoculation.

The stem inoculation was performed by modifying the stem-wound method developed by Ghimire et al. (2019). At the second trifoliate stage (V2), a prick was made with an autoclave-sterilized 200 µL pipette tip at approximately 50 mm below the first trifoliate node, and a mycelial plug with a diameter of 0.6 cm from a fresh colony of 7-day-old *D. longicolla* isolate was pressed against the wound, with the mycelial side facing the wound. The plants were then transferred to humidity chambers maintained at a temperature of 24±3ºC and a humidity of 90%. After an incubation period of 7 days, the plants were rated for stem infection based on the following scale: 0 = healthy plant without infection, 0.5 = elongated lesion along the stem with a lesion length greater than 1 cm, 1 = dead plants (Ghimire et al. 2019) (Figure 3.1).

Figure 3.1: Disease rating was conducted at 7 days post inoculation based on the scale: 0=healthy plant without symptom (left), 0.5= stem with lesion and extending over 1cm but no plant dead (center), 1= dead plants (right) (Ghimire et al. 2019). Image credit: Ghimire et al. (2019).

Seed resistance experiment under laboratory conditions

The seed inoculation method modified from Petrovic et al. (2021) was used for conducting the seed infection experiment. For each accession, the inoculum consisted of a solution prepared by scraping mycelial tissue from two 7-day-old culture plates and combining it with 25 mL of

autoclaved distilled water. Following surface sterilization with 0.5% sodium hypochlorite for 45 s, 70% ethanol for 1 min, rinsing twice with autoclaved distilled water, and air drying under a hood, 16 seeds per accession were immersed in 25 mL of the inoculum and incubated for 4 h. After the incubation period, four seeds per accession were taken randomly from 16 seeds and placed on a 2% agar plate supplemented with 0.4 g/L of streptomycin sulfate to avoid any bacterial contamination and 1 mL of the inoculum was added to each seed to ensure proper infection. The plates were then sealed with Para-film and incubated at a temperature of 23±2ºC under diffused light conditions. The experiment was conducted using a CRD, with eight replications per experiment per accession, and was repeated once. Each seed served as one replication. Four seeds were incubated per Petri plate. After a 7-day incubation period, the seeds were evaluated by modifying the scale developed by Zhao et al. (2022b): 0 = no symptoms and seeds germinated, 1 = mild symptoms (slight discoloration of seeds and seeds germinated), 2 = obvious lesions (seeds were diseased and germinating), and 3 = severe lesions (cotyledons found to be rotting) (Figure 3.2).

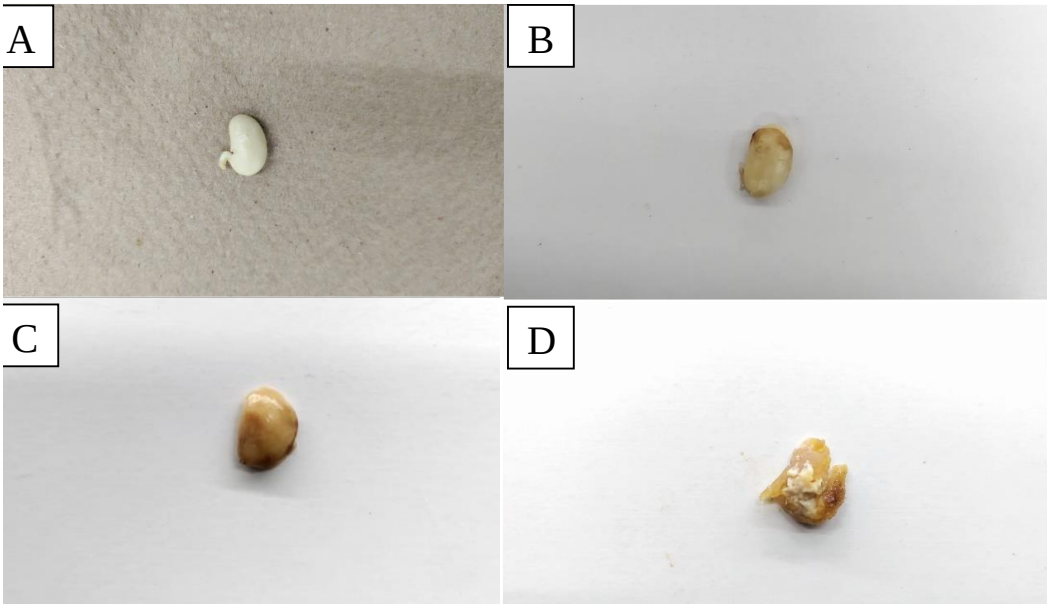

Figure 3.2: Disease rating of seed decay was recorded seven days post inoculation based on scale: 0= no symptoms and seeds germinated (A), 1 = mild symptoms (slight discoloration of seeds and seeds germinated) (B), 2 = obvious lesions (seeds were diseased and germinating) (C), and 3 = severe lesions (cotyledons found to be rotting) (D) (Zhao et al. 2022b).

Statistical analyses

For both stem and seed experiments, the Fligner-Killeen test for homogeneity of variances (Conover et al.1981) was conducted to determine if disease rating data from two experiments could be combined using 'car' package in R (version 4.3.2) at α=0.05. The homogeneity of variance test was satisfied for stem (*P*=0.54) and seed infection (*P*=0.98) experiments and hence, the two experiments were combined for further analysis.

Since the disease rating data was ordinal in nature, ANOVA-type statistics (Shah and Madden 2004) was performed for stem and seed infection separately using nparLD package (Noguchi et al. 2012) in R. The relative treatment effects (RTEs) were calculated from the mean rank as

$$\hat{p} = \frac{1}{N}\left(\bar{R}_i - \frac{1}{2}\right) \quad (2)$$

where $\bar{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$, where $\bar{R}_i$ is the mean rank for the i^{th} treatment and R_{ik} is the rank of Xi_k (the measurement in the k^{th} replication of treatment i for N^{th} observation, N is the number of observations) (Shah and Madden 2004). Relative Treatment Effects (RTEs) of each accession were compared with susceptible checks using 95% confidence intervals (CIs). If a 95% CI of an accession came within the same range as the susceptible check, we considered that there were no significant differences in the disease severity of these accessions.

Correlation analysis was also performed between RTEs of stem and seed disease ratings of accessions using Spearman's rank correlation test (Turechek 2004) in R at α=0.05. For assessing the correlation results, when Spearman's correlation coefficient (r) was 0, it was regarded as no correlation, 0 to 0.39 indicated weak correlation, 0.40 to 0.69 indicated moderate correlation, and 0.70 to 1 indicated strong correlation (Mohan et al. 2023).

Results

Stem resistance experiment under greenhouse conditions

A significant effect (ATS= 26.26; DF=7.59; P<0.004) of genotypes was observed on disease severity (expressed as RTE) caused by the *D. longicolla* isolate on soybean stems. Twenty-nine accessions originated from Germany (PI 417507), China (PI 407749, PI 567381 B, PI 612715, PI 603756), South Korea (PI 398697, PI 398752), Japan (PI 417361), South Africa (PI 417561), Taiwan (PI 504488), the United States (Barnes, Vinton 81, Blackhawk, PI 547453, Williams 82, PI 689003, PI 547652, PI 642055, PI 547432, PI 591491, PI 547610, PI 591490, DB06×0006-93, Jupiter-R, Bay, AP 55, and Tracy-M), Pakistan (PI 371611) and Uruguay (PI 235335) showed significantly low disease severity compared to Hawkeye (Table 3.1). When reactions of genotypes to stem infection were compared with Williams 82, a total of 15 genotypes (PI 417507, Barnes, Blackhawk, PI 603756, PI 547432, PI 547453, PI 398697, PI 398752, PI

504488, PI 689003, DB06×0006-93, PI 591490, PI 591491, PI 417561, and PI 407749) were found less susceptible. This was performed since Williams 82 was used as a susceptible check in screening PI lines to stem infection by *D. longicolla* in Kontz et al (2016), and in Li et al. (2010a) and in Mengistu and Reddy (2005).

Seed resistance experiment under laboratory conditions

A significant effect of genotypes was observed on the disease severity (ATS= 8.16; DF= 7.17; $P<0.003$) caused by *D. longicolla* isolate on soybean seeds. Six accessions, onefrom China (PI 407749) and five from the United States (Hawkeye, Vinton 81, Bay, PI 547453, DB06×0006-93), were significantly less susceptible than PI 371611 based on 95% confidence intervals (Table 3.1). When compared to Williams 82, none of the genotypes were found to be less susceptible than seed infection caused by *D. longicolla.*

Correlation analyses

The correlation coefficient between the RTEs of accessions from the stem and seed experiment was not significant and weakly correlated ($r=0.15$, $P=0.3$).

Table 3.2: Relative Treatment effects (RTEs) and corresponding 95% confidence interval to assess the effect of 39 soybean genotypes on the disease rating of seed decay and stem infection caused by *D. longicolla*.

Maturity Group	Accession	State and /or Country of Origin	Relative treatment effect (95% Confidence Intervals)	
			Stem infection [a]	Seed decay [b]
0	PI 417507	Germany	0.31 (0.24,0.39) *	0.40 (0.33, 0.48)
0	Barnes	North Dakota, United States	0.28 (0.23, 0.34) *	0.52 (0.40,0.63)
I	PI 612715	Heilongjiang Sheng, China	0.50 (0.36, 0.64) *	0.44 (0.32,0.56)
I	Vinton 81	Iowa, United States	0.35 (0.25, 0.46) *	0.32 (0.30,0.34) *
I	Blackhawk	Iowa, United States	0.31 (0.24, 0.38) *	0.35 (0.29,0.41)
I	10049-142-31	Mississippi, United States	0.74 (0.64, 0.81)	0.65 (0.51,0.77)
II	PI 603756	China	0.25 (0.24, 0.26) *	0.42 (0.32,0.53)
II	PI 547453	Illinois, United States	0.25 (0.24, 0.26) *	0.34 (0.29,0.40) *
II	Hawkeye 63	Illinois, United States	0.74 (0.64, 0.81)	0.51 (0.38,0.65)
II	Hawkeye [c]	Iowa, United States	0.84 (0.77, 0.89)	0.32 (0.30,0.34) *
III	Williams 82	Illinois, United States	0.55 (0.45, 0.65) *	0.35 (0.30,0.40)
III	PI 398697	Chungcheongnam-do, South Korea	0.26 (0.24,0.26) *	0.56(0.44,0.67)
III	PI 398752	Chungcheongnam-do, South Korea	0.25 (0.24,0.26) *	0.47 (0.36,0.58)
III	PI 417361	Japan	0.66 (0.60, 0.71) *	0.68 (0.57,0.77)
III	PI 504488	Taiwan	0.25 (0.24,0.26) *	0.68 (0.60,0.75)
III	PI 689003	Mississippi, United States	0.28 (0.23, 0.33) *	0.78 (0.67,0.86)
III	PI 547652	Illinois, United States	0.46 (0.34, 0.58) *	0.54 (0.41,0.66)
III	DB06×0006-93	Mississippi, United States	0.25 (0.24, 0.26) *	0.32 (0.30,0.34) *
III	PI 548298	China	0.68 (0.58, 0.76)	0.56 (0.44,0.67)
IV	PI 158765	China	0.69 (0.67,0.70)	0.44 (0.35,0.54)

Table 3.2: Relative Treatment effects (RTEs) and corresponding 95% confidence interval to assess the effect of 39 soybean genotypes on the disease rating of seed decay and stem infection caused by *D. longicolla* (continued).

Maturity Group	Accession	State and /or Country of Origin	Relative treatment effect (95% Confidence Intervals)	
			Stem infection [a]	Seed decay [b]
IV	PI 235335	Uruguay	0.43 (0.28, 0.57) *	0.71 (0.55,0.83)
IV	PI 652443	Missouri, United States	0.78 (0.65, 0.87)	0.76 (0.58,0.89)
IV	PI 642055	Mississippi, United States	0.57 (0.46, 0.67) *	0.40 (0.31,0.50)
IV	PI 547432	Illinois, United States	0.25 (0.24, 0.26) *	0.56 (0.42,0.68)
IV	PI 591491	Illinois, United States	0.25 (0.24, 0.26) *	0.35 (0.29,0.42)
IV	PI 547610	Illinois, United States	0.64 (0.52, 0.73) *	0.36 (0.29,0.44)
IV	PI 591490	Illinois, United States	0.28 (0.23, 0.34) *	0.38 (0.30,0.47)
IV	PI 417561	South Africa	0.25 (0.24, 0.26) *	0.54 (0.42,0.65)
IV	AP 350	United States	0.74 (0.62, 0.83)	0.41 (0.33,0.50)
IV	PI 371611[d]	Pakistan	0.64 (0.56, 0.70) *	0.53 (0.40,0.65)
V	PI 381668	Uganda	0.90 (0.85, 0.93)	0.47 (0.37,0.57)
V	PI 407749	Shanghai Shi, China	0.25 (0.24, 0.26) *	0.32 (0.30,0.34) *
V	PI 567381 B	Shaanxi Sheng, China	0.65 (0.56, 0.73) *	0.44 (0.34,0.54)
V	Bay	Virginia, United States	0.61 (0.50,0.71) *	0.32 (0.30,0.34) *
V	AP 55	United States	0.66 (0.60,0.71) *	0.75 (0.69,0.80)
V	11043-211-10	Mississippi, United States	0.72 (0.60, 0.82)	0.5 (0.38,0.60)
VI	Tracy-M	Mississippi, United States	0.59 (0.48, 0.69) *	0.62 (0.51,0.72)

Table 3.2: Relative Treatment effects (RTEs) and corresponding 95% confidence interval to assess the effect of 39 soybean genotypes on the disease rating of seed decay and stem infection caused by *D. longicolla* (continued).

Maturity Group	Accession	Relative treatment effect (95% Confidence Intervals)	
		Stem infection [a]	Seed decay [b]
VII	Bragg	Florida, United States	0.71 (0.59, 0.80)
IX	Jupiter -R	Florida, United States	0.52 (0.42, 0.63) *

[a] Disease rating data was analyzed using nonparametric statistics (Shah and Madden 2004) and expressed as relative treatment effect. The asterisk '*' represents less susceptible genotypes compared to susceptible check Hawkeye based on 95% confidence intervals.

[b] Disease rating data was analyzed using nonparametric statistics (Shah and Madden 2004) and expressed as relative treatment effect. The asterisk '*' represents less susceptible genotypes compared to susceptible check PI 371611 based on 95% confidence intervals.

[c] Susceptible check of stem experiment.

[d] Susceptible check of seed experiment.

Discussion

In this study, 39 accessions were evaluated for their reaction to a single isolate of *D. longicolla* as both a seed and a stem pathogen. The stem experiment was conducted in the greenhouse by using the stem wound inoculation method (Ghimire et al. 2019) and the seed experiment was performed under laboratory conditions by modifying the Petrovic et al. (2021) inoculation method. Among 39 genotypes, 29 were less susceptible to *D. longicolla* as a stem pathogen when compared to the susceptible check 'Hawkeye'. In contrast, six genotypes were found less susceptible to *D. longicolla* as a seed pathogen than susceptible check PI 371611. Five genotypes (Vinton 81, PI 547453, DB06×0006-93, PI 407749 and Bay) were observed to have low disease severity for both seed and stem infections. There was a weak non-significant correlation (P=0.3) between stem infection and seed infection caused by *D. longicolla* across the screened soybean genotypes, suggesting that stem and seed responses to this fungus may be controlled by distinct genetic loci.

In the stem experiment, among the 29 genotypes identified to be less susceptible to *D. longicolla* when compared to Hawkeye, PI 417507 was reported as less susceptible by Kontz et al. (2016) and Mohan et al. (2023). In this study, fifteen accessions were found to be less susceptible to stem infection by *D. longicolla* when compared to Williams 82. The isolate used in this study was reported to be aggressive in the isolate aggressiveness study performed by Mohan et al. (2023). They reported a significant effect of isolate-by-accession on disease severity on the accessions including PI 417507 and Hawkeye by multiple isolates of *D. longicolla.* Mohan et al. (2023) was the first study to report the difference in virulence of *D. longicolla* isolates and their effect on host. PI 612715 was found resistant to stem infection by *D. longicolla* in this study compared to Hawkeye. However, it was previously identified as susceptible to stem infection by *D. longicolla* compared to Williams 82 genotype (Kontz et al. 2016). When the reaction of PI 612715 was compared to that of Williams 82 in this study, susceptible reaction of PI 612715 was evident which aligns with results from Kontz et al. (2016).

In the seed experiment, the modified inoculation method, adapted from Petrovic et al. (2021) was employed. Initially, the seed inoculation method developed by Petrovic et al. 2021 was used however, in this study, this method did not produce infection on the susceptible cv. PI 371611 which may be deciphered as the difference in isolates of *D. longicolla* used in these studies (17-DIA-35 vs 17-OP-DIA-SOY-054), and difference in susceptible genotypes (cv. 'Sava' vs PI 371611). We could use neither 17-DIA-35 isolate nor cv. 'Sava' in this study due to their unavailability. When the cv. PI 371611 was used as the susceptible check, six genotypes were found less susceptible to seed infection caused by *D. longicolla.* This includes PI 407749 which had a significantly lower percent seed infection than susceptible check PI 417420 in Li et al. (2015). However, PI 398697, PI 398752, PI 417361, PI 504488, PI 158765, PI 235335, and PI

381668 identified as resistant by Li et al. (2015), were observed were found to be susceptible in this study. Accessions PI 398752, PI 398659, PI 504488, and PI 158765 were found resistant in non-inoculated plots in Arkansas, Missouri, and Mississippi whereas PI 235335, PI 417361, and PI 381668 were identified resistant in both inoculated and non-inoculated plots in three states (Li et al. 2015). We speculate that the difference in response of these accessions in this study, and the Li et al. (2015) studies may be attributed to differences in screening environment (field vs. lab), growth stages (V2 in the greenhouse vs. R8 (full maturity) growth stages in the field), type of inoculum (conidia during artificial infection and possibly in natural inoculum vs. mycelia), and the environmental factors affecting disease development (temperatures above 20°C, relative humidity above 90% vs. prolonged precipitation for five days followed by delayed harvest due to late season rains in 2009 and 2012 field trials). Thus, developing and comparing inoculation methods using multiple isolates of *D. longicolla* to screen seed resistance in both field and lab conditions is essential.

Isogenic genotypes refer to genotypes that are genetically identical or nearly identical except for specific traits under study. In other words, they are nearly homozygous lines that have been selected to have very similar genetic backgrounds. In our study, we included six isolines of cultivar Clark (PI 591491, PI 591490, PI 547610, PI 547432, PI 547652 and PI 547453) which have similar genetic backgrounds, except in having different sets of 'E' maturity genes. All lines had a susceptible reaction to *D. longicolla* stem infection compared to Hawkeye, although all lines except PI 547453 had low disease severity of seed infection by *D. longicolla* compared to PI 371611. These lines had been screened for their effect of maturity on Diaporthe seed decay along with isogenic lines of cultivar Harosoy (Gillen et al. 2012). The isogenic lines of Clark were found to have more percent seed infection than isogenic lines of Harosoy in the field trials

conducted in 2004 and 2005 in Stoneville, MS and the study identified no effect on maturity for the incidence of Diaporthe seed decay in these two sets of isogenic lines (Gillen et al. 2012).

The correlation coefficient between disease severity (expressed as RTE) caused by *D. longicolla* as a seed pathogen and stem pathogen showed a weak association. This indicates the possibility that genes conferring resistance to *D. longicolla* as a seed pathogen and stem pathogen may not be similar. There might be two reasons that help to explain the results obtained. First, the host's defense mechanism against infections on seed and stem by *D. longicolla* might be different. We suggest this hypothesis based on the study by Underwood and Misar (2024), who reported differences in resistance to stem and leaf infection by *D. helianthi* Muntañola-Cvetkovic et al. and *D. gulyae* Shivas et al., the causal agents of Phomopsis stem canker in sunflower (*Helianthus annuus* L.). The authors concluded that the resistance to stem canker and leaf infection caused by these pathogens might be under independent genetic control, which aligns with results found by Degener at al. (1999). Second, the resistance mechanism to stem and seed infections might be coordinated by respective tissues. A study conducted by Haidoulis and Nicholson (2022) investigated the transcriptome response in *Bradypodium distachyon* (L.) Beauvois, a model plant to study Fusarium head blight and root rot caused by *Fusarium graminearum* Schwabe by performing RNA-seq analysis and differential gene expression in both *B. distachyon* and *F. graminearum*. The results showed genes associated with signalling various genes related to resistance reaction were differentially expressed during head blight and root rot in *B. distachyon* and identified a change in transcriptome expression of effectors of *F. graminearum* was dependent as well as independent of tissue it infects. In our study, this can be evaluated by screening a large number of soybean genotypes (>200), with diverse genetic backgrounds, and performing genome-wide association mapping studies to determine common quantitative trait

loci, single nucleotide polymorphisms, and putative candidate genes for seed and stem resistance to *D. longicolla*. Further investigations are required to confirm these preliminary findings by screening the susceptibility reaction to stem infection and seed decay caused by *D. longicolla* using large number of diverse soybean genotypes.

The findings of this study contribute valuable information regarding the presence of parental materials in USDA soybean germplasm, thereby aiding breeders in the development of genotypes that are resistant to *D. longicolla*, a pathogen affecting both seeds and stems. Furthermore, the variability observed in the susceptibility of soybean accessions previously determined to be resistant to seed and/or stem infection by *D. longicolla* emphasizes the need to determine effective inoculation methods and favorable environmental conditions (temperature, relative humidity) favoring for disease development using multiple isolates. Moreover, the role of soybean pods in causing seed infection by *D. longicolla* should be investigated, which might play an important role in the incidence of Diaporthe seed decay during late reproductive stages of the crop. Consequently, it is recommended that future studies include a larger number of soybean genotypes containing segregating germplasm to identify the specific genes and mechanisms involved in the stem and seed resistance to *D. longicolla*.

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CHAPTER FOUR: GENERAL CONCLUSIONS

To manage *Diaporthe* diseases, soybean growers are recommended to adopt integrated disease management strategies which include cultural practices such as conventional tillage, crop rotation with non-host crops, weed management, utilizing resistant cultivars, prophylactic foliar fungicide application, and timely harvest of the crop. This thesis dealt with two important aspects of disease management measures; (1) evaluating the fungicide sensitivity of *D. aspalathi, D. caulivora* and *D. longicolla* collected from different soybean U.S states to one of the QoI fungicides, azoxystrobin and (2) determining the different forms of resistance to *D. longicolla* as a stem and seed pathogen in soybean (*Glycine max* L.).

In the first study, a mycelial growth inhibition assay was performed to determine the sensitivity of 75 isolates of *D. aspalathi* , *D. caulivora* , and *D. longicolla* collected from 16 U.S states from 2014 to 2022, to azoxystrobin. ANOVA-type statistics showed a significant effect of isolates on the effective concentration at which the mycelial growth was inhibited by 50% (EC_{50}) for *D. aspalathi* (P=0.002), *D. caulivora* (P=0.014), and *D. longicolla* (P=0.003). A significant difference in EC_{50} among *D. aspalathi* isolates was observed, however, no significant difference in EC_{50} was observed among isolates of *D. caulivora* and isolates of *D. longicolla.* Even though frequency distribution graphs plotting EC_{50} of the isolates tested showed a rightly skewed distribution (P<0.001for *D. aspalathi*, P<0.0014 for *D. caulivora* and P<0.002 for *D. longicolla* when normality test was conducted), since we do not have enough number of isolates and no trend was observed related to year of collection and EC_{50} of isolates in corresponding year in each species, we cannot confirm a shift in the sensitivity of *Diaporthe* species in soybean to azoxystrobin. From this objective, we recommend precaution when using quinone outside inhibitor (QoI) fungicides solely for the prophylactic application to control foliar diseases in

soybean, as non- targeted spray would also expose *Diaporthe* spp. and other fungal pathogens in soybean, resulting in a selection pressure over sensitive and insensitive fungal individuals, favouring the latter. A need-based rotation of fungicides with different modes of action is recommended. Frequent monitoring of the sensitivity of *Diaporthe* spp. in soybean to azoxystrobin is also warranted.

The second objective explores different forms of resistance against *D. longicolla* as a stem and seed pathogen in 39 soybean genotypes. A significant effect of genotypes was observed on disease severity caused by stem ($P<0.004$) and seed ($P<0.005$) infection by *D. longicolla*. A total of 29 genotypes were found less susceptible to stem infection compared to susceptible check Hawkeye. However, only six genotypes had significantly low disease severity for seed infection compared to check PI 371611. Five genotypes were found less susceptible to both stem and seed infection caused by *D. longicolla*. Although our sample size is not sufficient to confirm a relationship between stem and seed infection caused by *D. longicolla*, we suggest a difference in host resistance mechanism against stem and seed infection caused by *D. longicolla* considering this study on a preliminary basis. Future studies such as genome-wide association studies (GWAS), and transcriptome expression analyses with more than 200 genotypes are essential to confirm this relationship and would be helpful in breeding programmes in future.

Overall, this research contributed to developing management strategies to control selected species of *Diaporthe* causing diseases in soybeans.