MOLECULAR CHARACTERIZATION AND PATHOGENICITY

OF

SUNFLOWER STEM PATHOGENS

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Molecular characterization and pathogenicity of sunflower stem pathogens

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ABSTRACT

Sunflower (*Helianthus annuus* L.) production can be limited by several stem diseases. Among these, Phomopsis stem canker causes frequent yield reductions in Australia, Europe and North America. In the U.S., while *Diaporthe helianthi* was assumed to be the sole causal agent, *Diaporthe gulyae* was found to cause Phomopsis stem canker in Australia. In order to determine the causal agent in the U.S., 234 isolates were cultured from 275 infected sunflower stems collected from the Northern Great Plains. Phylogenetic analyses of the internal transcribed spacer region, elongation factor subunit 1-α, and actin gene sequences confirmed two species, *D. helianthi* and *D. gulyae*. Four methods were tested to assess the Phomopsis stem canker response using four *D. helianthi* isolates on sunflowers. Stem-wound method was adopted for subsequent experiments based on the recovery of *D. helianthi* and its correlation with disease severity at 14-d after inoculation. Aggressiveness of two *Diaporthe* species was determined in greenhouse and results suggest they did not vary significantly (p=0.0012) in their aggressiveness, except at 3-d after inoculation. Among the nine genotypes screened for resistance, USDA 'PI 162784' and 'PI 219649' were less susceptible to the two *Diaporthe* spp.

Fusarium is commonly regarded a minor pathogen on sunflowers in most production regions of the world. A total of 110 *Fusarium* isolates were recovered from 1,637 stalks randomly sampled for stem diseases in the Northern Great Plains and identified to species level. Phylogenetic analyses of repetitive-sequence-based polymerase chain reaction fingerprints and the translation elongation factor 1-alpha gene revealed that *Fusarium* isolates from sunflowers represented clades of eight species; namely, *F. graminearum*, *F. proliferatum*, *F. culmorum*, *F. avenaceum*, *F. oxysporum*, *F. acuminatum*, *F. sporotrichioides* and *F. equiseti*. Pathogenicity studies of eight *Fusarium* spp. in the greenhouse suggests *F. sporotrichioides* and *F. equiseti*

were most aggressive. The study comparing the aggressiveness of three *Fusarium* spp. and *V. dahliae* isolates representing six VCGs showed *V. dahliae* VCG4B and VCG2A were significantly more aggressive ($p \leq 0.05$) than *F. sporotrichioides, F. oxysporum* and *F. equiseti*. The identification of *Diaporthe* spp. and *Fusarium* spp. on sunflowers has implications for breeding for resistance and disease management.

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Thank you.

Febina M. Mathew
North Dakota State University
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DEDICATION

To my beloved mom and dad …..............for their love, care, and sacrifices

To my loving sister and brother…..........for keeping me strong and alive

To my advisors ……....................................for believing in me and my abilities

To my friends ……......................................for their support and understanding

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INTRODUCTION

History of sunflowers

Cultivated sunflower (*Helianthus annuus* L.) is one of the few crops that originated in North America. *H. annuus* belongs to the family Compositae (Asteraceae) and is one of 67 species in the genus *Helianthus*. Some archaeologists suggest that sunflower may have been domesticated prior to corn (*Zea mays* L.) by American Indians between 4,500 and 3,000 years ago (Diamond, 1999). Following its domestication, sunflower was used by American Indians as an important source of edible seeds as well as for ornamental and ceremonial use (Seiler and Rieseberg, 1997, Heiser *et al*., 1969). Around 1500 A.D., sunflower was brought to Europe by Spanish explorers where it became an ornamental plant in gardens (Putt, 1997). From Spain, sunflower spread rapidly through France and Italy initially, and continued north and east into Europe. Before 1,800 A.D., sunflower reached Russia and became recognized as a source of vegetable oil. Late in the 19th century, these Russian-bred cultivars made the trip back to North America. It wasn't until the 1950's that the sunflower became an important agronomic crop in the United States (U.S.), with cropping beginning in North Dakota and Minnesota as a result of commercial interest in the production of sunflower oil. By the 1970s, with the development of hybrids sunflower farming spread into South Dakota, Kansas, and other states including Nebraska, Texas and California.

Pre-Columbian archaeological sites in Mexico have suggested the possibility of a second independent domestication center (Heiser, 1998). Blackman *et al*. (2011) looked at 60 sunflower populations from the U.S., Mexico and Canada and analyzed the sequence diversity of three domestication genes (*c4973*, *HaFT1*, and *HaGA2ox*) and additional neutral markers. Their study identified patterns of genetic diversity in Mexican sunflowers (domesticated and wild) consistent with all other domesticated varieties known to have originated from an eastern North American

domestication site. Thus, the current sunflower populations in both the U.S. and Mexico descend from a lineage of eastern U.S. sunflowers, probably in the area of present-day Arkansas.

Production of sunflowers

At present, most U.S. commercial sunflower production is in the western and upper Great Plains states of Colorado, Kansas, Minnesota, Nebraska, North Dakota, South Dakota and Texas (Sandbakken and Kleingartner, 2007), with North Dakota and South Dakota being the leading producers of sunflowers in the U.S (USDA-NASS, 2014). Though sunflower has often been referred to as a 'minor' crop in the U.S., the crop was valued at nearly $450 million with an average yield of 1,378 pounds per acre in 2013 (USDA-NASS, 2014).

The geographic expansion of sunflower production internationally has occurred from the use of high-oil varieties and hybrids. Today, in addition to the U.S., production is also significant in Europe, Russia, Ukraine, Argentina, China, India, Turkey and South Africa. According to United States Department of Agriculture (USDA, 2014), world sunflower production was 39 million metric tons in 2013, with European Union, Russia, Ukraine, Argentina, United States, China, India and Turkey being the top sunflower producers.

Sunflower types and uses

The sunflower is one of the four most important oil crops around the world and is grown on over 61 million acres (25 million hectares) worldwide (National Sunflower Association, 2014). Sunflower seeds typically contain about 45 to 53 percent oil and 15 to 18 percent protein (Skorić, 2009). In addition to oil and protein, sunflower kernels contain tocopherols, minerals, and vitamins.

There are two types of sunflower hybrids: (1) the oilseed type, and (2) the confection or non-oilseed type. Oilseed types produce relatively smaller, black seeds in a thin hull that adheres

tightly to the achene. The end use of oilseed sunflower is oil that is used primarily for human consumption although it is also suitable for biofuels. The non-oilseed type produces the large, striped seeds that are used for human food snacks in the shell or as kernels, in baking ingredients, and in birdseed blends.

Seeds of sunflower are mainly used for their oil content, which accounts for 80% of its value in the world (Žilić *et al*., 2010). Sunflower oil comprises around 8% of the total vegetable oil production in the world. The vegetable oil market is dominated by palm oil (± 33 percent), soybean oil (± 29 percent) and rapeseed oil (± 16 percent) (International Trade Centre, 2011). The by-product of sunflower biodiesel is glycerin, which can be used in the manufacture of soap or other products. Breeding efforts in recent years has led to the production of hybrid sunflowers with different oil profiles (types of saturated and unsaturated fats) (Skorić, 2009). The categories are (1) standard or linoleic, (2) NuSun or mid-oleic, and (3) high oleic. Among the commercial sunflower hybrids, both mid-oleic and high-oleic hybrids have found the widest application in commercial production including vegetable-oil based fuel and food coatings.

Sunflower production practices

The time required for development of a sunflower plant and the time between the various stages of development depends on the genetic background of the plant and the growing season. Almost all commercial varieties of sunflower are hybrids, and these are selected on the basis of high yield with high seed oil content (at least 40 percent), a test weight of at least 25 pounds per bushel, and disease and insect resistance (if available). Maturity is typically reached within 2200-2300 growing degree days after planting sunflowers or 120-150 days into the field season. A standardized growth stage scheme was created for sunflowers into vegetative and reproductive stages by Schneiter and Miller (1981) (Table 1.1).

Table 1.1. Growth stages of sunflower plant (Schneiter and Miller, 1981)

Stage	Description
Vegetative Emergence (VE)	Seedling has emerged and the first leaf beyond the cotyledons is less than 4 centimeter (cm) long.
Vegetative Stages (V-number) (For example, V-1, V-2, etc.)	These are determined by counting the number of true leaves at least 4 cm in length.
Reproductive Stages (R-1)	The terminal bud forms a miniature floral head rather than a cluster of leaves.
R-2	The immature bud elongates 0.5 to 2.0 cm above the nearest leaf attached to the stem.
R-3	The immature bud elongates more than 2 cm above the nearest leaf.
R-4	The inflorescence begins to open.
R-5 (decimal) (For example, R-5.1, R-5.2, etc.)	This stage is the beginning of flowering and can be divided into sub- stages dependent upon the percent of the head area that have completed or are in flowering.
R-6	Flowering is complete and the ray flowers are wilting
R-7	The back of the head has started to turn a yellow.
R-8	The back of the head is yellow but the bracts remain green.
R-9 (Physiological maturity)	The bracts become yellow and brown.

Soils

Sunflowers grow best on well drained soils with a neutral pH (6.5-7.5) (Berglund, 2007). One novel usage of sunflower has been to 'bioremediate' polluted soil. Sunflowers have been used in the removal of toxic waste from the environment such as removal of uranium using rhizofiltration in the remediation of ground water (Lee and Yang, 2010).

Crop rotation

A proper crop rotational sequence is recommended for sunflowers to help reduce pathogen inoculum levels in the soil, allow for pesticide rotation, manage overwintering insect populations, weeds, water usage and fertility management. Disease pressure can be exacerbated by failure to rotate fields and/or by too close a sequencing of sunflower in the practiced rotations. For instance, in the Northern Great Plains, Sclerotinia stalk and head rot (white mold) can cause a high level of yield loss, and other broadleaf crops are hosts to the same pathogen (Bolton *et al*., 2006). Thus,

rotations of at least four years including cereals, are typically recommended to help manage diseases such as Sclerotinia stalk and head rot.

Fertilizers

Fertilizer applications should be made based on a soil test (Franzen, 2010). Based on the recommendations from soil test, nitrogen applications can be made pre-plant, at seeding, post-seeding or a combination of these methods. Phosphorous and potassium may also be applied in fall or spring before a tillage operation. For instance, the fertility required for a 2,000-pound sunflower crop typically consists of 100 pounds of nitrogen and 50 pounds of phosphorus and potassium, depending on the results of soil samples. Deficiencies of iron, manganese, zinc, copper, molybdenum, boron and chlorine are not common in the Northern Great Plains but can appear in other sunflower production regions (Berglund, 2007).

Water management

Sunflower may be considered a highly drought tolerant crop, but its extensively branched, deep taproot aids the plant during water stress. Consequently, sunflowers have historically been produced on ground not suitable to other, more water-needy crops, such as soybeans. In the Northern Great Plains, sunflower production is largely dry-land, even in the more arid areas in the Western Dakotas. However, in the even more arid High Plains, production is often irrigated (High Plains Integrated Pest Management Guide, 2009).

Planting dates

Sunflower seeding usually begins any time after May 15 and is completed by June 15 in the Northern Great Plains. However, because sunflowers are more resilient to frost than many crops, particularly legumes, they can be planted later than recommended. In late springs in the Northern Great Plains, sunflower acreage is likely to exceed planting predictions.

Plant populations

Seeding rate for sunflowers depends on sunflower type. For instance, oil sunflower populations range from 20,000 to 22,000 plants/acre, while confection sunflowers do not typically exceed 18,000 plants/acre to ensure large seed size (Berglund, 2007).

Plant row spacing

The majority of sunflower fields in the Northern Great Plains are planted in narrow row spacing (19 to 38 cm) for both confection and oil sunflowers (Kandel, 2012). Sunflower fields with row spacing greater than 50 cm are common in the other sunflower production regions (Kandel, 2012).

Plant depth

Sunflowers are planted in moisture and the ideal seeding depth is 3.80 to 5.08 cm deep (High Plains Integrated Pest Management Guide, 2009).

Tillage

The tillage regimes practiced in the Northern Great Plains include conventional, minimum and zero-tillage (Kandel, 2012). However, factors to be considered when deciding what tillage regime to utilize include soil type, climate, fertilizer regime and rotation.

Weed management

Sunflowers are planted at low densities and do not cover the ground early enough to prevent early weed establishment. Weeds that most often present problems for sunflower growers in Northern Great Plains include; kochia (*Kochia scoparia* L.), redroot pigweed (*Amaranthus retroflexus* L.), Canada thistle (*Cirsium arvense* L.), common lambsquarters (*Chenopodium album* L.), and wild buckwheat (*Polygonum convolvulus* L.) (Kandel, 2012). Weed competition with sunflowers during the first four to six weeks after emergence can reduce sunflower yield by 30%.

Thus, good production practices should include a combination of cultural and chemical methods to manage weeds. The introduction of 'Clearfield' and 'ExpressSun™' herbicide tolerant sunflower hybrids has given growers new options for post-emergence control of broadleaf and grass weeds. Both systems require pre-emergence herbicide treatments (such as 'Spartan' (Sulfentrazone) or 'Prowl' (Pendimethalin)) to combat key grass and broadleaf weeds during the crop season (Zollinger, 2014).

Pest management

Sunflowers are challenged by a number of pests including insects, weeds and diseases. Fields should be monitored frequently to determine pest species present and if populations are at economic threshold levels. Integrated Pest Management (IPM) can be used to manage pests by combining biological, cultural, and chemical tools to minimize economic, health, and environmental risks. A number of factors influence the abundance and diversity of pests on sunflowers from year to year. Integration of different pest management tools can minimize pest numbers and the cost of management without unnecessary crop losses. The utilization of multiple management tools is particularly important for management of sunflower diseases where at least two pathogens have multiple races that may overcome resistance, at least one pathogen has fungicide insensitivity to one FRAC (Fungicide Resistance Action Committee) group, and several pathogens cannot be managed by a single tool (Gulya *et al*., 2013).

Harvest

Sunflowers are usually one of the last crops to be harvested in the fall since fall frosts help in drying down the crop. Sunflowers are often harvested when the seed moisture is below 16 percent. Harvesting sunflowers when seed moisture is greater than 16 percent can result in scuffing during harvesting, shrinkage during drying and molding in storage (Berglund, 2007).

Diseases as a limiting factor in sunflower production

Sunflower production can be limited by many diseases, which significantly reduce the yield and quality under optimal conditions for disease development. More than 90 sunflower diseases have been reported worldwide (Gulya *et al*., 1997). The severity of losses induced by plant pathogens is often related to the crop growth stage at the time of disease onset and this can affect the yield. Thus, understanding the factors that trigger the development of disease epidemics, anticipating that development and rapid identification of disease problems that are occurring is critical when creating and implementing effective strategies for disease management.

In the U.S., the most common diseases of sunflower are caused by fungi and oomycetes these include rust (*Puccinia helianthi* Schwein.), downy mildew (*Plasmopara halstedii* (Farl.) Berl. & de Toni), Verticillium wilt (*Verticillium dahliae* Kleb.), Sclerotinia stalk and head rot (*Sclerotinia sclerotiorum* (Lib.) de Bary), Phoma black stem (*Phoma macdonaldii* Boerema), Rhizopus head rot (*Rhizopus* spp.), Charcoal rot (*Macrophomina phaseolina* (Tassi) Goid) and Phomopsis stem canker (*Diaporthe* spp.) (Kandel, 2012). When available, the most economical and effective management of sunflower diseases is the planting of hybrids resistant to these pathogens. Using a rotation scheme that allows a minimum of three to four years between successive sunflower crops is effective in reducing inoculum and managing diseases. Hybrids with resistance to some races of *P. helianthi, P. halstedii*, *V. dahliae*, and other disease pathogens are available (Gulya *et al*., 1997). Additionally, seeds may be treated with fungicides to control seedborne pathogens, such as *P. halstedii*. Fungicides are labeled for control and/or management of diseases, such as rust, and Sclerotinia white mold in North Dakota (Friskop *et al*., 2014). Recent work suggests that while rust can be effectively and economically managed by a foliar application

(Friskop *et al*., 2014), applications for the management of Sclerotinia head rot are neither effective nor economically viable (Wunsch *et al*., 2014).

Phomopsis stem canker

Description

Phomopsis stem canker is a fungal disease which has been responsible for high yield losses in sunflower crop in the main production regions of the world (Gulya *et al*., 1997, Masirevic and Gulya, 1992). In the U.S., the disease was identified in Ohio in 1980 (Herr *et al*., 1983), followed by Texas in 1982 (Yang *et al*., 1984), and Minnesota and North Dakota in 1984 (Hajdu *et al*., 1984). According to the annual survey coordinated by the National Sunflower Association (NSA), disease severity has increased from approximately 1.5% of the crop in 2001 to 24.4% in 2012 (Kandel, 2012). Yield losses occur if plants lodge during seed fill due to stem weakness caused by pith damage. The disease typically causes significant losses in yield (10-50%) and in oil content (10-15%) when the environmental conditions are favorable (Laville, 1986).

Phomopsis stem canker was attributed to *Diaporthe helianthi* M. Muntanola-Cvetkovic *et al.* (Muntanola-Cvetkovic *et al.*, 1985) when the disease was first described from Voivodina region of the former Yugoslavia in 1980 (Muntanola-Cvetkovic *et al*., 1985). The sexual state is characterized by perithecia that are produced on cortical tissues. The perithecia are long necked, spherical to globular, yellowish to black and 290 to 430 micrometer (µm) in diameter. Asci are globular to sub-cylindrical, 60 to 76.5 µm long by 8.7 to 12.5 µm wide. Ascospores are two-celled, ellipsoidal and constricted at the septum, 15 to 17.5 µm long by 8.7 to 12.5 µm wide (Muntanola-Cvetkovic *et al*., 1985, Udayanga *et al*., 2011). The asexual state of *D. helianthi* is characterized by ostiolate, black pycnidia (120 - 290 µm in diameter) containing elongate, cylindrical phialides with well-developed collarettes that may produce two types of hyaline, non septate conidia: one-

celled α-conidia that are biguttulate, fusiform, and easily germinate on artificial media, and β-conidia that are filiform and rarely germinate (Rehner and Uecker, 1994, Muntanola-Cvetkovic *et al*., 1985, Wehmeyer, 1933). Generally, conidiophores are hyaline, branched and occasionally they are short and 1–2 septate (Muntanola-Cvetkovic *et al*., 1985, Udayanga *et al*., 2011). Asexual fruiting bodies, called pycnidia, are produced on stem and leaf lesions during the disease cycle. Pycnidia are globular, 120 to 290 µm in diameter, dark brown, ostiolate and submersed in tissue (Wehmeyer, 1933, Uecker, 1988, Muntanola-Cvetkovic *et al*., 1985, Maric and Masirivic, 1980). Although the asexual state is most commonly encountered on hosts under natural conditions, given the nomenclatural transition to one genus name for both sexual and asexual states of fungi (Wingfield *et al*., 2012) and nomenclatural priority by date over *Phomopsis* (Sacc.) Bubák (1905), we use *Diaporthe* Nitschke (1870) throughout this paper when referring to species or groups of isolates.

The identification of the causal agent of Phomopsis stem canker was a controversial matter since it was first identified in the former Yugoslavia in 1980 (Muntanola-Cvetkovic *et al*., 1985). Muntañola-Cvetkovic´ *et al*. (1985) found that multiple *Diaporthe* species were associated with cankers on sunflower in the former Yugoslavia, although only *D. helianthi* was responsible for the serious disease outbreaks. Aćimović and Štraser (1982) and Masirevic and Gulya (1992) determined differences in the symptoms caused by *D. helianthi* isolates on sunflowers, development of pycnidia, dimensions of pycnidia and conidia, type of conidia, temperature requirements for conidial development and formation of perithecia. Gulya *et al*. (1997) suggested that pathogenic *Diaporthe* species on sunflower might consist of more than one species or biotype with apparent biological differences between the isolates from Europe and the U.S. The U.S. isolates readily produce perithecia in culture while European isolates do not. Until a more precise

taxonomic identification could be applied to the causal agent of Phomopsis stem canker, researchers agreed that more than one species or biotype might be involved (Gulya *et al*., 1997).

Historically, host association has often been the basis for species identification in *Diaporthe*, as morphological and culture characteristics are unreliable for species differentiation (van Rensburg *et al*., 2006). For resolving taxonomic issues, molecular phylogenies derived from DNA sequence analyses of the ribosomal internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA genes, translation elongation factor-1α (EF-1α) gene region, actin (ACT) gene region, and mating-type (MAT) genes have been used (Thompson *et al*., 2011, Udayanga *et al*., 2011, Ash *et al*., 2010, Santos *et al*., 2010). It is also now recognized that host is of minor importance for identification of *Diaporthe* species (Mostert *et al*., 2001, Rehner and Uecker, 1994). Moreover, recent studies have demonstrated that a number of *Diaporthe* species have wide host ranges (Gomes *et al*., 2013, Udayanga *et al*., 2011, Ash *et al*., 2010, Santos and Phillips, 2009), and more than one *Diaporthe* species can occur on a single host (Santos and Phillips, 2009). *Diaporthe* species are known to infect many Compositae hosts that may act as alternative hosts or inoculum reservoirs for the pathogen. For instance, although cocklebur (*Xanthium* sp.) was found to be a host of a species of *Diaporthe* that were pathogenic on sunflower (Carriere and Petrov, 1990), Mihaljcevic and Vukovevic (1994) concluded that sunflower was the major source of inoculum of *D. helianthi* in a study of 14 genera of weeds in Yugoslavia.

Hyde *et al*. (2010) suggested that discarding the host-based species concept is required for the development of a useful and reliable classification for *Diaporthe* and highlighted that there had been much confusion around the application of the name *D. helianthi*, particularly with the quarantine and trade issues. While *D. helianthi* was assumed to be the sole causal agent of the disease in sunflowers, some researchers have confirmed multiple *Diaporthe* species involved in

Phomopsis stem canker disease development in Croatia (Vrandecic *et al*., 2009) and Australia (Thompson *et al*., 2011). As of 2014, a total of eight *Diaporthe* species have been documented as responsible for Phomopsis stem canker on sunflowers across the world, namely, *Diaporthe helianthi* M. Muntanola-Cvetkovic *et al.* (Muntañola-Cvetković *et al.*, 1985), *Diaporthe gulyae* Shivas, Thompson and Young (Thompson *et al*., 2011), *Diaporthe kongii* Shivas, Thompson and Young (Thompson *et al*., 2011), *Diaporthe kochmanii* Shivas, Thompson and Young (Thompson *et al*., 2011), *Diaporthe stewartii* A. L. Harrison (Mathew *et al*., 2012), *Diaporthe longicolla* (Hobbs) J. M. Santos, Vrandečić & A. J. L. Phillips (Mathew *et al*., 2012), *Diaporthe novem* J. M. Santos, Vrandečić & A. J. L. Phillips (Thompson, personal communication), and *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. (Cooke and Ellis, 1878). Of these, *D. helianthi*, *D. gulyae*, *D. longicolla* and *D. stewartii* have been reported causing Phomopsis stem canker on sunflowers in the U.S., specifically in the Northern Great Plains (Mathew *et al*., 2012).

Disease cycle

The pathogen over-winters as mycelium on plant debris and produces perithecia (fruiting bodies) under warm, moist conditions the following year. Temperatures of 20°C to 30°C are optimal for ascospore development and subsequent infection (Masirevic and Gulya, 1992). Lack of air movement due to dense plant stands and closed leaf canopy can also favor infection. The disease cycle begins with ascospore production and release from perithecia. Ascospores infect the margins of older sunflower leaves via guttation droplets where moisture accumulates. Spores are spread among plants by splashing rain and irrigation water. Following infection, mycelium invades intercellular spaces and terminal veinlets, spreading in a systemic fashion to larger branches of the water conducting system, reaching the midrib and ultimately the leaf petiole and stem. In general, it takes 25-30 days from leaf infection until a stem lesion is formed. Frequent rainfall from budding

(growth stage R1) stage onwards accelerates Phomopsis stem canker development, especially during periods of extended high temperature and high humidity (Maric *et al*., 1988). Pycnidia may form in diseased stem tissue and when mature, release asexual conidia (pycniospores). Two types of conidia, α-conidia and/or β- conidia may be formed in the stem. While it is not known whether α-conidia contribute significantly to new cycles of infection, β- conidia do not cause secondary infection (Mihaljcevic *et al*., 1985). Instead, perithecia are the principal source of inoculum, releasing ascospores in response to environmental conditions throughout the growing season (Gulya, personal communication). Ascospores are wind-borne and may travel considerable distances in air currents, from weed hosts, sunflower fields or from crop to crop. The pathogen survives between sunflower crops in infested crop debris (Masirevic and Gulya, 1992).

Symptoms and damage

Severity of Phomopsis stem canker depends on susceptibility of the sunflower hybrid, plant growth stage at the time of infection and environmental conditions (Masirevic and Gulya, 1992). Leaf lesions initially appear as brown, irregularly shaped spots with a yellow chlorotic margin at the edges of leaves. Leaf veins and petioles darken into brown to black cankers that later become ashy gray around the diseased petiole base. Individual lesions eventually coalesce and cause death of the entire leaf. The infection spreads through the margins of sunflower leaves where moisture accumulates, eventually reaching the lateral veins and the main stalk, where lesions can block the water and nutrient flows leaving the stem hollow. Stem lesions develop a wet appearance, increase in length often reaching 15 to 20 cm, and eventually girdle the stalk, whereupon plants wilt and lodge. Small black pycnidia containing asexual conidia form on the stem lesions. The fungus produces overwintering structures (perithecia) on the infected crop residues, which makes it

difficult to manage. Thus, tillage practices that encourage rapid residue breakdown will minimize the risk to following crops (Masirevic and Gulya, 1992).

Management

Tillage. Crop residue left on the soil surface facilitates the development of *Diaporthe*. Burying crop residues by tillage can be effective to reduce disease but the rate of residue breakdown will also be influenced by weather conditions (Mihaljcevic *et al*., 1985).

Rotation. *Diaporthe* can survive in crop residues for up to five years, depending on weather conditions and tillage practices. A rotational break of non-hosts between two and four years is effective in inoculum reduction. In the intervening years, non-host crops such as small grains and corn (*Zea mays* L.) are preferentially sown (Masirevic and Gulya, 1992).

Plant density and nitrogen fertilization. Dense plant stands should be avoided as this can result in taller plants with thin stems making them more prone to lodging following infection. A thick canopy, which can result from dense plant stands or excessive use of nitrogen, should be avoided since this favors environmental conditions for disease. Debaeke and Moinard (2010) studied the effects of crop management (plant density, nitrogen fertilization, and irrigation) and genotypic tolerance (susceptible and tolerant cultivars) in a two year study. Their study showed that early *Diaporthe* infection is related to changes in microclimate resulting from crop management practice or cultivar architecture. The number of girdling lesions per plant was highest with high N fertilization compared to crops receiving little or no fertilization (Debaeke and Moinard, 2010). Thus, managing crop density and nitrogen fertilization may help decrease the incidence and severity of stem canker.

Clean seed. *Diaporthe* species can be seed-borne so it is important to ensure that the sunflower seeds used for planting are pathogen free (Masirevic and Gulya, 1992).

Biological control. No biological control strategies have been developed for management of Phomopsis stem canker in the U.S (Masirevic and Gulya, 1992).

Chemical control. Chemical control has been used in Europe as a management tool for Phomopsis stem canker but are not commonly used in the United States. Fungicide evaluations were attempted in 2009 in the United States, but disease failed to develop (Markell *et al*., 2010). In 2011, a natural Phomopsis stem canker epidemic occurred in a fungicide-rust trial (Mathew *et al*., 2012), and treatments with fungicides applications of Proline (Prothioconazole) and Tebuzol (Tebuconazole) in the trial significantly reduced the disease incidence. Similarly, seed yield was significantly higher in treatment plots where fungicides were applied than the non-treated plots. However, rust pressure was also reduced under fungicide applications, so the direct yield impact that Phomopsis stem canker had on yield was unclear (Mathew *et al*., 2012).

Genetic resistance. Resistance to *D. helianthi* has been found in sunflower germplasm (Skoric, 1985) and in descendants from inter-specific crosses between sunflower and wild *Helianthus* (Besnard *et al*., 1997, Griveau *et al*., 1992). Initial genetic studies revealed that the control of resistance to *D. helianthi* is oligogenic (Vranceanu *et al*., 1993). Further studies have suggested that resistance to *D. helianthi* is quantitative in nature and governed by mostly additive gene action (Viguié *et al*., 1999). Hybrid registration procedures in France requires disease evaluations for several diseases and thus hybrids resistant to Phomopsis stem canker is publicized (Centre Technique Interprofessionnel des Oléagineux Métropolitains (CETIOM) annual publication). Currently, there are no commercial sunflower hybrids in the U.S. with resistance to these *Diaporthe* species, although 30 parental lines from Europe and Russia have been identified with potential resistance to *D. helianthi* (Talukder *et al*., 2014, Gulya, personal communication).

Fusarium stem disease

Description

Species of *Fusarium* are commonly reported as endophytes, epiphytes, saprophytes, and pathogens of various economically important crops. They are distributed worldwide, from the temperate to the tropical regions (Leslie and Summerell, 2006). The widespread distribution of *Fusarium* species may be attributed to the ability of these fungi to grow on a wide range of substrates and their efficient mechanisms for dispersal (Burgess, 1981). In North Dakota, *Fusarium* species have a wide host range, ranging from dry edible beans (Bilgi *et al.*, 2008), field peas (Mathew *et al.*, 2008), to potato (Estrada Jr. *et al*., 2010), sugar beet (Rivera *et al*., 2008), and small grains (Burlakoti *et al.*, 2007).

For sunflowers, *Fusarium* is considered a minor pathogen in most places of the world, including U.S. production areas (Gulya *et al*., 1997). However, in Russia, *Fusarium* has become a serious problem for sunflowers since the 1990s (Gontcharov *et al*., 2006). In the Krasnodar region of Russia, 12 different species of *Fusarium* were found during surveys in 1999-2001 (Antonova *et al*., 2002). All the isolated *Fusarium* species were determined to be pathogenic, causing various symptoms. *Fusarium sporotrichioides* Sherb. was the most aggressive of the species recovered. In the U.S., Fusarium wilt of sunflower was first reported in Texas caused by unidentified *Fusarium* spp. (Orellana, 1971). In 2009, *F. sporotrichioides* and *Fusarium acuminatum* Ellis and Everhart were reported causing stem disease along with *Fusarium oxysporum* Schlecht. Emend. Snyd & Hans. in Minnesota (Mathew *et al*., 2010). Although the economic implications of *Fusarium* spp. are unclear in the U.S., the pathogens are currently a serious economic problem on sunflower in Russia, where yield losses up to 80% has been reported. In India, yield losses up to 45% caused by Fusarium wilt of sunflower has been reported (Aćimović, 1998).

The most common *Fusarium* species identified as sunflower pathogens worldwide are *F. oxysporum, F. solani* (Martius) Appel & Wollenweber emend. Snyder & Hansen*, F. verticillioides* (Saccardo) Nirenberg*, F. equiseti* (Corda) Saccardo*, F. culmorum* (W. G. Smith) Saccardo*, F. sporotrichioides* and *F. semitectum* Berkeley & Ravenel (Tančić *et al*., 2012, Mathew *et al*., 2010, Antonova *et al*., 2002, Nahar and Mushtaq, 2006, Nahar and Mushtaq, 2007). *Fusarium* spp. is characterized by macroconidia and microconidia from slender phialides. Macroconidia are hyaline, two- to several-celled, fusiform- to sickle-shaped, mostly with an elongated apical cell and pedicellate basal cell (Leslie and Summerell, 2006). Microconidia are one- to two-celled, hyaline, pyriform, fusiform to ovoid, straight or curved. Chlamydospores may be present or absent (Leslie and Summerell, 2006). Although sexual and/or asexual state can be commonly encountered on hosts under natural conditions, given the nomenclatural transition to one genus name for both sexual and asexual states of fungi (Wingfield *et al*., 2012) and the application of the name was preserved for *Fusarium* (Geiser *et al*., 2013), we use "*Fusarium*" throughout this paper when referring to species or groups of isolates.

Identification of *Fusarium* species is often difficult due to the variability among isolates of the same species and between species, for example, shape and size of conidia and colony color on a common microbiological media and because morphological features that are required for identification are not always well developed such as the presence or absence of macroconidia in some isolates. The translation elongation factor 1-α (EF1-α) gene is typically used for molecular identification of *Fusarium* species because it is highly informative at the species level in *Fusarium* (Geiser *et al*., 2004). In addition, non-orthologous copies of the gene have not been detected in the genus *Fusarium*, and universal primers are available that work across the phylogenetic breadth of the genus (Geiser *et al*., 2004, O'Donnell *et al*., 1998). More recently, molecular tools such as

restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR)-based fingerprinting with primers matching enterobacterial repetitive intergenic consensus (ERIC) sequences and repetitive extragenic palindromic (REP) elements, and restriction fragment analysis of PCR-amplified ribosomal intergenic spacers (IGS) have been used for the genetic characterization of *F. oxysporum* strains (Edel *et al*., 1995).

The wilt pathogen, *F. oxysporum*, is the most economically important species in the genus, given its cosmopolitian distribution and numerous hosts (Leslie and Summerell, 2006). Disease development caused by *F. oxysporum* is favored by high temperatures and warm moist soils. Optimal temperature for the growth of *F. oxysporum* on artificial media is between 25˚C and 30˚C, while optimal soil temperature for root infection by this pathogen is 30˚C or above (Leslie and Summerell, 2006). In a pathogenicity study of 12 *Fusarium* spp. on sunflower plants in Pakistan by Nahar and Mushtaq (2006) and Nahar and Mushtaq (2007), the typical symptoms were wilting, collar rots, stem rots and seedling rots, damping-off, stunting, yellowing, tip burning and reduction in growth. Among these symptoms produced by *Fusarium* spp., wilting and seedling rot were found to be the most important (Nahar and Mushtaq, 2006, Nahar and Mushtaq, 2007). Plants inoculated with *F. chlamydosporum* Wollenw. & Reinking, *F. equiseti, F. acuminatum* Ell. and Ev., *F. solani* and *F. subglutinans* Wollenw. & Reinking showed the highest degree of wilting, whereas the highest levels of seedling rot was observed by *F. sporotrichioides, F. oxysporum* and *F. solani* (Nahar and Mushtaq, 2006, Nahar and Mushtaq, 2007).

Interactions, and/or confusion, with other pathogens complicate *Fusarium* spp., as they are also known to form mixed infections with *Macrophomina phaseolina* (Tassi) Goid., on common hosts such as sunflowers under favorable weather conditions resulting in charcoal rot and wilt (de Barry, 1985, Orellano, 1970, Gulya *et al*., 2010, Mathew *et al*., 2010). As a result of mixed

infections, sunflower plants with charcoal rot symptoms in the field usually have not only *M. phaseolina* present but also *F. oxysporum* in the stem, which can cause the sunflower plants to eventually wilt and die (de Barry, 1985). Symptoms of Fusarium wilt are known to be confused with Verticillium wilt (*Verticillium dahliae* Kleb) in other crops (Egel and Martyn, 2007) since the leaves turn yellow and the vascular system is discolored in both diseases.

Disease cycle

Fusarium is a genus of common soil pathogen that infect the roots of the sunflower plants by means of mycelia or by germinating spores penetrating the plant's root tips, lateral roots or through root wounds. The pathogen can also spread short distances by water splash or long distances by infected seeds (Egel and Martyn, 2007). Once within the plant, the fungus grows and multiplies in the vascular system of the roots. The fungus produces asexual spores (macroconidia and microconidia) that enter into the sap stream and are transported upward in the plant. The spores germinate and clog the vascular vessels, preventing the plant from taking up and translocating nutrients. Toxic substances are believed to be secreted due to the interaction of the fungus and the host plant (Egel and Martyn, 2007). The plant transpires more than it can transport as a result of which the leaves wilt, and the plant eventually dies. Wilt symptoms typically are not observed until the fungus has colonized the underground parts of the plant (Egel and Martyn, 2007).

Resting structures (chlamydospores) are formed within infected plant parts. After the host plant dies, the fungus survive as mycelia or chlamydospores in the soil debris or crop residues. Chlamydospores are stimulated to germinate by exudates from the roots of the sunflower plants which they then infect. Once contact is made with a new plant host, the fungus again invades the underground parts, progresses upward, and the cycle is repeated. Soil moisture and pH have little

effect on Fusarium wilts, as the fungus can thrive in a wide range of soil types (Egel and Martyn, 2007).

Symptoms and damage

Disease and symptom development are extremely dependent upon air and soil temperatures. Symptoms are most severe at constant temperatures of 29°C to 32°C. Typical symptoms of Fusarium wilt include a drooping and yellowing of the leaves, often starting on one side, and stunting of the plant (Bhargava *et al*., 1978). Lower parts of the stem are dark and discolored, always in the pith and sometimes on the outside. When infected stems are split, brown to black streaks are evident in the vascular system (Bhargava *et al*., 1978). Masses of white or pinkish *Fusarium* spores (conidia) are formed in fruiting bodies, called sporodochia, on the surface of infected or dead stems, usually near the soil line (Egel and Martyn, 2007) .

Management

Strategies to manage *Fusarium* are limited by the ability of the fungi to survive in soil for long periods, with or without a host plant, and the colonization of the vascular tissues within a plant. There are currently limited management strategies available for Fusarium on sunflowers in the U.S. However, on other crops such as vegetables, fruits, field crops, trees, shrubs, and ornamentals where Fusarium wilt is a problem (Anonymous, 1988), disease management strategies described below are taken to reduce the effects of the disease:

Tillage. Crop residue left on the soil surface fosters development of *Fusarium*. Burying crop residues by tillage can be effective to reduce disease but the rate of residue breakdown will also be influenced by weather conditions (Anonymous, 1988).

Weed management. Good weed management is important to reduce pathogen populations of *Fusarium* when sunflowers are planted, as many weed species are alternative hosts of *Fusarium* (Anonymous, 1988).

Clean seed. *Fusarium* species can be seed-borne so it is necessary to ensure that the sunflower seeds used for planting are disease free. *Fusarium* contamination can also be limited by taking care not to transfer infected residues on equipment and vehicles during planting (Anonymous, 1988).

Biological and chemical control. No biological or chemical control is available for Fusarium stem disease in the U.S. Fungicidal seed treatments are commonly used in sunflowers, but the primary target is typically *P. halstedii* (Friskop *et al*., 2014), and little efficacy data on *Fusarium* exists.

Genetic resistance. Resistance to *Fusarium* has been identified in sunflower inbred lines in Russia (Gontcharov *et al*., 2006). However, commercial sunflower hybrids in the U.S. with known tolerance or resistance to *Fusarium* spp. do not exist.

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CHAPTER ONE. MOLECULAR CHARACTERIZATION OF *DIAPORTHE* SPECIES INFECTING SUNFLOWERS IN THE UNITED STATES AND OTHER COUNTRIES

Abstract

Phomopsis stem canker frequently causes yield reductions on sunflowers (*Helianthus annuus* L.) on several continents, including Australia, Europe and North America. Between 2001 and 2012, the incidence of Phomopsis stem canker has increased 16 fold in the Northern Great Plains of the U.S. *Diaporthe helianthi* has been assumed to be the sole causal agent of the disease in the U.S.; however, a recently characterized pathogen, *Diaporthe gulyae* Shivas, Thompson and Young was found to the primary cause of Phomopsis stem canker in Australia and linked to an increase of disease incidence in that country. This prompted a re-evaluation of the causal agent in the United States. The objectives of this study were to (i) characterize the *Diaporthe* species causing stem canker on sunflowers in the Northern Great Plains; and (ii) compare the *Diaporthe* species present in the U.S. with those in other important sunflower growing areas in the world. To determine the identity of *Diaporthe* spp., 234 isolates were cultured from 275 infected sunflower samples collected from the North Central Great Plains and 27 isolates were cultured from 65 stems received from international collaborators. Phylogenetic analyses of DNA sequences of the ribosomal DNA internal transcribed spacer region, elongation factor subunit 1-α, and actin gene regions, in comparison with those of type specimens, confirmed two species in the U.S., specifically *D. helianthi* (70% of the recovered isolates) and *D. gulyae* (30%). Phylogenetic analyses of DNA sequences of the ribosomal DNA internal transcribed spacer region from eighteen international isolates identified *D. helianthi* in Russia, Yugoslavia and Serbia, Bulgaria and Croatia and seven international isolates identified *D. gulyae* in Canada. While it is unclear if

the identification *D. gulyae* is related to an increase in Phomopsis stem canker in the United States, it has implications for breeding for resistance and disease management.

Introduction

Phomopsis stem canker is widespread in most sunflower (*Helianthus annuus* L.) growing regions with prolonged high temperatures and high rainfall of the United States (Gulya *et al*., 1997), Europe (Masirevic and Gulya, 1992) and Australia (Thompson *et al*., 2011). The disease was first described from the Voivodina region of the former Yugoslavia in 1980 and the causal agent was described as *Diaporthe helianthi* M. Muntanola-Cvetkovic *et al.* (Muntañola-Cvetković *et al.*, 1985). Phomopsis stem canker can cause significant losses in yield (10- 50%) and in oil content (10-15%) in Europe (Laville, 1986).

According to data from the annual survey coordinated by the National Sunflower Association (NSA), Phomopsis stem canker incidence in the United States (U.S.) has increased from approximately 1.5% of the crop in 2001 to 24.4% in 2012. Historically, yield and oil losses due to Phomopsis stem canker in the U.S. have been minimal, if occurring at all. However, in 2010, a Phomopsis stem canker epidemic occurred on sunflowers in the Northern Great Plains (Fig. 2.1) with incidence and severity highest in the Northern Great Plains states of North Dakota (ND), South Dakota (SD) and Minnesota (MN), where over 75% of the U.S. sunflower crop is grown. Several isolated fields in ND and MN had an incidence of over 100% of the plants infected and yield losses up to 40% (Markell and Gulya, personal communication).

Phomopsis stem canker has historically been attributed to *D. helianthi* (Muntañola-Cvetković *et al.*, 1985, Aćimović and Štraser, 1982). However, the possibility of multiple species infecting sunflowers was raised by the researchers previously in the early 1980s (Muntañola-Cvetković *et al*., 1985), but there was little evidence to support the hypothesis. At this time host

association was used for species identification in *Diaporthe*, as morphological and culture characteristics are inadequate or unreliable for species differentiation (van Rensburg *et al*., 2006). This supported the assumption that all *Diaporthe* strains isolated from sunflower are causative agents of the Phomopsis stem canker caused by *D. helianthi*. However, there appeared to be distinct biological differences between isolates of *Diaporthe* causing disease on sunflower in Europe and those causing disease on sunflower in the U.S. (Gulya *et al*., 1997). The U.S. isolates readily produced perithecia in culture but European isolates did not. Despite some evidence of biological differences among the *Diaporthe* isolates and a hypothesis suggesting multiple species were involved, disease symptoms associated with the pathogen on sunflowers were thought to be the same everywhere. More recently, there has been molecular evidence to suggest there are several species of *Diaporthe* that can cause Phomopsis stem canker on sunflower (Thompson *et al*., 2011). Three *Diaporthe* species, *Diaporthe gulyae* Shivas, Thompson & Young, *Diaporthe kongii* Shivas, Thompson & Young and *Diaporthe kochmanii* Shivas, Thompson & Young, were responsible for the disease outbreaks in Australia in 2009 (Thompson *et al*., 2011), where *D. helianthi* has not been confirmed.

The specific objectives of this study were to (a) molecularly characterize (multi-locus phylogenetic evaluation and mating type) isolates of *Diaporthe* from sunflowers causing Phomopsis stem canker in Minnesota (MN), North Dakota (ND) and South Dakota (SD); and (b) compare the *Diaporthe* species causing Phomopsis stem canker in the U.S. with those in other important sunflower growing areas in the world using sequence analysis of the internal transcribed spacer (ITS) region.

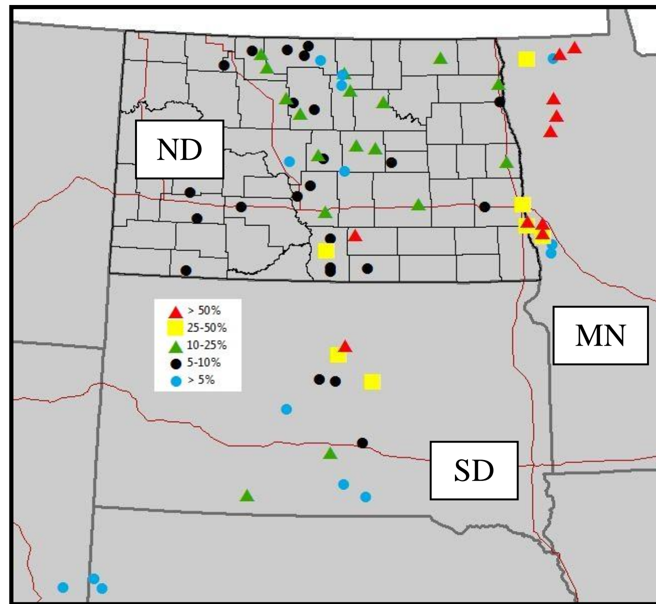

Fig. 2.1. Incidence (%) map of Phomopsis stem canker in the Northern Great Plains in the United States in 2010 (Courtesy: Dr. John Nowatzki and Dr. Tom Gulya).

Materials and method

Survey collection and identification of *Diaporthe* isolates

A survey of sunflower fields was conducted in the Northern Great Plains states of MN, ND and SD in an effort to collect isolates of all stem pathogens in 2010, 2011 and 2012. In 2010, 55 fields were selected arbitrarily, with no fields being closer than 5 km from a previously selected field (Table 2.1). In each field, one to two transects (rows) were randomly selected. In each transect in a field, two to three sunflower stalks exhibiting a variety of stem symptoms (wilting, lodging, and lesions of varying size and color) were arbitrarily selected until the end of the transect was reached. A total of 51 and 26 sunflower fields infected by Phomopsis stem canker were selected arbitrarily in 2011 and 2012 respectively and two to three diseased stalks were collected along one to two transects from these fields (Table 2.1). In total, 234 isolates (83 isolations in 2010, 96 in 2011, and 55 in 2012) were recovered from 275 stalks collected (Table 2.1).

Stem samples were washed with tap water for 2 min; and approximately 1 cm long pieces were cut from infected tissue. The pieces were surface sterilized in sodium hypochlorite (10%) and ethanol (70%) for 1 min each, rinsed in sterile distilled water four times and blotted between sterile filter papers. Four pieces were placed on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 0.02% streptomycin sulfate. Plates were incubated at room temperature for 7- to 14-d under 12-h of alternating light and dark conditions. Cultures were scored for presence or absence of different fungi, including *Diaporthe*, based on morphology (Barnett and Hunter, 1972). After 14-d, *Diaporthe* isolates were purified by transferring hyphal tips to fresh plates of PDA amended with 0.02% streptomycin sulfate.

Table 2.1. Number of fields surveyed by year, state, and Phomopsis stem canker incidence in fields where *Diaporthe* spp. was identified in the Northern Great Plains (MN, ND and SD) in 2010, 2011 and 2012

Year	State	Fields Surveyed	Total number of stems collected	Number of stems symptomatic of Phomopsis stem canker	Total number of isolates recovered	*Diaporthe* isolation (%)
2010	MN	1	6	4	4	66.67
2010	ND	6	8	7	7	87.50
2010	SD	48	76	72	72	94.74
2011	MN	14	37	27	27	72.97
2011	ND	14	23	8	8	34.78
2011	SD	23	70	61	61	84.29
2012	MN	10	26	26	26	100.00
2012	ND	5	14	14	14	100.00
2012	SD	11	15	15	15	100.00

In addition to 275 sunflower stalks collected during the 2010-2012 survey in the Northern Great Plains, a total of 65 stalks (34 in 2011 and 31 in 2012) were received from seven countries (Tables 2.2). Collaborators from the seven countries arbitrarily selected sunflower stalks either exhibiting symptoms including lesions of varying size and color or those that were consistent with

Phomopsis stem canker symptoms including elongated lesions, pith discoloration and wilting or lodging. Pathogen isolation techniques described above were used, and stems were autoclaved after isolations. Cultures were scored for presence or absence of different fungi (including *Diaporthe*) based on morphology (Barnett and Hunter, 1972). After 14-d, *Diaporthe* isolates were purified by transferring hyphal tips to fresh plates of PDA amended with 0.02% streptomycin sulfate. From the international collections, a total of 12 isolates were recovered from 34 stalks in 2011 (five from Russia and seven from Canada) and 15 isolates from 31 stalks in 2012 (two from Yugoslavia and Serbia, one from Bulgaria and 12 from Croatia) (Tables 2.2).

Table 2.2. Number of stalks received from international collaborators and number of *Diaporthe* isolates by country recovered in 2011 and 2012

Country	Year	Number of Fields	Number of Stalks	Number of isolates recovered
Russia	2011	7	7	5
Canada	2011	2	20	7
China	2011	3	7	0
Turkey	2012	3	7	0
Canada	2012	2	2	0
Yugoslavia and Serbia	2012	9	9	2
Bulgaria	2012	1	1	1
Croatia	2012	12	12	12

DNA of 234 isolates from the U.S. collected during the three-year survey and 27 isolates from international collaborators was extracted from lyophilized mycelium scraped from the surface of a 7-d culture growing on PDA and re-suspended in $50\mu L^{-1}$ of rehydration solution (1% TE buffer) using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). A 5-µl aliquot of each DNA sample was run electrophoretically on a 1% agarose gel to confirm quality. DNA was also quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The 234 *Diaporthe* isolates were identified to species by amplifying and sequencing the

internal transcribed spacer (ITS) regions using primers ITS1 and ITS4 (White *et al*., 1990). Cycle parameters included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (White *et al*., 1990). Forward and reverse sequences were edited and contigs were aligned using Bioedit (Hall, 1999). Analysis of the edited sequences was performed using Basic Local Alignment Search Tool nucleotide (BLASTN) searches at the GenBank nucleotide database (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). Fungi were identified based on top BLAST results (lowest e-value, highest score, and greatest similarity). Isolates that were found to have more than 95% identity with *Diaporthe* sequences in GenBank and have an e-value less than e-10 in the BLASTN searches were used for further analysis.

In order to establish a well-resolved phylogeny and clarify the phylogenetic position of *Diaporthe* sp. infecting sunflowers in the U.S., 19 representative *Diaporthe* isolates were randomly selected from the Northern Great Plains and characterized by phylogenetic analyses of three gene fragments including ITS, translation elongation factor 1-alpha (EF1-α) and actin (ACT) (Table 2.3). Nineteen representative isolates from international collaborators were sequenced using the ITS primers only (White *et al*., 1990, Table 2.4).

The intron region of the EF1-α gene was amplified using primers EF1-728F and EF1-986R (Carbone and Kohn, 1999). For the sequencing of partial actin gene, fragments of the ACT gene was amplified using the primers ACT-512F and ACT-783R (Carbone and Kohn, 1999). Reactions for all PCR amplifications were performed in a 25-µl mixture containing 20-30 ng/reaction of template DNA, 10 µM of each primer, 10 mM of each dNTPs, 5 units/µl of Taq DNA Polymerase (Qiagen, Valencia, CA), and 10x Qiagen PCR Buffer containing 15 mM $MgCl_2$ (Qiagen, Valencia,

CA). The PCR cycling protocols were: denaturation at 94°C for 5 min followed by 39 cycles of 30 s at 95°C, 50 s at 55°C (for primer pairs ACT-512F/ACT783R) or 58°C (for primers pairs EF1-728F/EF1-986R), 1 min at 72°C, and a final step of 10 min at 72°C (Carbone and Kohn, 1999). A 5-µl aliquot of each PCR product was run electrophoretically on a 1% agarose gel to confirm amplification. All DNA samples were sequenced (McLab, San Francisco, CA and GenScript USA Inc., Piscataway, NJ) using the respective PCR primers.

Table 2.3. Isolates of *Diaporthe* spp. originating from the U.S. used for phylogenetic study

Isolates[a]	Year of isolation	State	Species identity[b]
D6	2010	SD	*D. gulyae*
D9	2010	SD	*D. gulyae*
D12	2010	SD	*D. gulyae*
D14	2010	SD	*D. gulyae*
D21	2010	SD	*D. gulyae*
D32	2010	SD	*D. gulyae*
D40	2010	SD	*D. gulyae*
R_P131	2010	MN	*D. helianthi*
R_P129	2010	ND	*D. helianthi*
R_P132	2011	MN	*D. helianthi*
R_P126	2011	MN	*D. helianthi*
R_P121	2011	MN	*D. helianthi*
R_P105	2011	MN	*D. helianthi*
R_P140	2011	MN	*D. helianthi*
R_P137	2011	MN	*D. helianthi*
R_P134	2012	MN	*D. helianthi*
R_P118	2012	MN	*D. helianthi*
R_P139	2012	MN	*D. helianthi*
R_P107	2012	MN	*D. helianthi*

[a] Isolates are a subset of 234 isolates, and were chosen as representatives for species-level identification.
[b] Species identity was established based on phylogenetic analysis of the internal transcribed spacer region (ITS), elongation factor subunit 1-α (EF1α) and actin (ACT), conidial dimensions, and colony growth.

Table 2.4. International isolates of *Diaporthe* spp. used for phylogenetic study

Isolates[a]	Year of isolation	Country/ State	Species identity[b]
D6_WC	2011	Canada	*D. gulyae*
D9_WC	2011	Canada	*D. gulyae*
D12_WC	2011	Canada	*D. gulyae*
D14_WC	2011	Canada	*D. gulyae*
D21_WC	2011	Canada	*D. gulyae*
D32_WUS	2010	United States/ SD	*D. gulyae*
D40_WUS	2010	United States/ SD	*D. gulyae*
R_P131_WR	2011	Russia	*D. helianthi*
R_P129_WR	2011	Russia	*D. helianthi*
R_P132_WR	2011	Russia	*D. helianthi*
R_P126_WY	2012	Yugoslavia and Serbia	*D. helianthi*
R_P121_WY	2012	Yugoslavia and Serbia	*D. helianthi*
R_P105_WR	2011	Russia	*D. helianthi*
R_P140_WR	2011	Russia	*D. helianthi*
R_P137_WB	2012	Bulgaria	*D. helianthi*
R_P134_WUS	2012	United States/ MN	*D. helianthi*
R_P139_WUS	2012	United States/ MN	*D. helianthi*
R_P118_WUS	2012	United States/ MN	*D. helianthi*
R_P107_WUS	2012	United States/ MN	*D. helianthi*

[a] Isolates are a subset of 27 isolates, and were chosen as representatives for species-level identification.
[b] Species identity was established based on phylogenetic analysis of the internal transcribed spacer region (ITS), conidial dimensions, and colony growth.

Molecular phylogenetics

The ITS, EF1-α and ACT sequences of *Diaporthe* isolates were aligned using the default parameters of ClustalX (Thompson *et al*., 1997) and adjusted manually by visual examination using the Molecular Evolutionary Genetics Analysis (MEGA) software v5 (Tamura *et al*., 2011) prior to being exported as NEXUS files for subsequent analyses. Prior to the combined analyses, the concordance of the three gene datasets was evaluated with the partition-homogeneity test (Farris *et al*., 1994) implemented with PAUP* v4.0b10 (Sinauer Associates, Inc., Sunderland, MA; Swofford, 2002), using 1,000 random repartitions (Felsenstein, 1985), with MAXTREES set to

5,000. The null hypothesis of congruence was rejected if $p<0.001$ (Darlu and Lecointre, 2002, Dettman *et al*., 2003).

For individual and combined analyses, the ML phylogeny was estimated with Bayesian inference (BI) with MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) under the estimated model of evolution. For BI analyses, two simultaneous runs were conducted with Markov chain Monte Carlo (MCMC) chains with default priors and a conservative burn-in of 25% and tree sampling every 100 generations. Four parallel MCMC chains were run for 1,000,000 generations; a burn-in of 2500 generations was found to be sufficient to achieve stationary model parameters using Tracer v1.4.1 (Rambaut and Drummond, 2007). Within each run, the average standard deviation of split frequencies (ASDSF) and the potential scale reduction factor (PSRF) statistics from MrBayes were used to evaluate topological and branch-length convergence, respectively.

The best-fitting evolutionary models for the Bayesian analyses were estimated using ModelTest v3.7 (Posada and Crandall, 1998) by Akaike information criterion (AIC) (Akaike, 1974). For the ITS region, the general time reversible (GTR) model with a proportion of invariant sites (+I) and a six-category, discrete gamma (+G) shape distribution (Lanave *et al*., 1984) was selected as the best fitting model in ModelTest. The outgroup *Valsa ceratosperma* (Tode) Maire was obtained from GenBank (NCBI Accession number AY347335). For the combined EF1-α and ACT gene phylogenetic analyses, the best fitting model of nucleotide substitution was the HKY+G (Yang, 1994) and Transversion (TVM) +G was selected as the best fitting model in ModelTest. The outgroup *Chrysoporthella hodgesiana* Gryzenhout & M.J. was obtained from GenBank (NCBI Accession number GQ290152 for *C. hodgesiana* EF1-α sequence and GQ290170 for ACT sequence). The 46 sequences in the combined data set (including the outgroup *C. hodgesiana*) comprised 719 bp of aligned sequence.

The Bayesian probabilities (PP) for each node were estimated from the resulting 50% majority-rule consensus tree and nodes with 95% or greater PPs was considered significant (Wilcox *et al.*, 2002). Each of the Bayesian MCMC analyses was run at least twice to confirm the consistency of the results. Phylogenetic trees inferred using MrBayes analyses were observed in FigTree v1.3.1 (Rambaut, 2009).

Mating type identification of the U.S. *Diaporthe* isolates

The amplification reactions for MAT1-1-1 and MAT1-2-1 genes were performed for 19 representative U.S. isolates following the protocol of Santos *et al.* (2010) using primers MAT1-1-1F, MAT1-1-1R, MAT1-2-1F and MAT1-2-1R (Table 2.4). All PCR products were visualized under ultraviolet light in agarose gels stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA) at a final concentration of 0.25×.

Results

Survey collection and identification of *Diaporthe* isolates

During the 3-year survey, two species of *Diaporthe* were isolated from infected sunflower stems sampled from the Northern Plains in the United States (Table 2.5). The frequency of *D. helianthi* and *D. gulyae* varied over the years among the three states. In 2010, *D. helianthi* was isolated at frequencies of 3.9% from samples collected in SD to 66.7% from samples collected in MN and 87.5% from samples collected in ND (Table 2.5). However, *D. gulyae* was isolated at frequency of 90.8% from samples collected in SD. In 2011, *D. helianthi* was isolated at frequencies of 73.0% from samples collected in MN and 34.8% ND to 84.3% in SD, while *D. gulyae* was isolated at frequency of 2.9% from samples collected in SD (Table 2.5). In 2012, only *D. helianthi* was isolated and at frequencies of 100% from samples collected in all the three states (Table 2.5).

Table 2.5. Isolates of *Diaporthe* spp. originating from the U.S. characterized into species using the ITS gene region

				Number of *Diaporthe* isolates recovered			
Year	State	Fields Surveyed	Total number of stems collected	*D. helianthi*	*D. helianthi* isolation (%)	*D. gulyae*	*D. gulyae* isolation (%)
2010	MN	1	6	4	66.7	0	0.0
2010	ND	6	8	7	87.5	0	0.0
2010	SD	48	76	3	3.9	69	90.8
2011	MN	14	37	27	73.0	0	0.0
2011	ND	14	23	8	34.8	0	0.0
2011	SD	23	70	59	84.3	2	2.9
2012	MN	10	26	26	100.0	0	0.0
2012	ND	5	14	14	100.0	0	0.0
2012	SD	11	15	15	100.0	0	0.0

Table 2.6. Isolates of *Diaporthe* spp. received from international collaborators (in 2011 and 2012) and characterized into species using the ITS gene region

Country	Year	Number of Fields	Number of Stalks	Number of *Diaporthe* isolates recovered			
				D. helianthi	*D. helianthi* isolation (%)	*D. gulyae*	*D. gulyae* isolation (%)
Russia	2011	7	7	5	71.4	0	0.0
Canada	2011	2	20	0	0.0	7	35.0
China	2011	3	7	0	0.0	0	0.0
Turkey	2012	3	7	0	0.0	0	0.0
Canada	2012	2	2	0	0.0	0	0.0
Yugoslavia and Serbia	2012	9	9	2	22.2	0	0.0
Bulgaria	2012	1	1	1	100.0	0	0.0
Croatia	2012	12	12	12	100.0	0	0.0

From the international collections, the frequency of *D. helianthi* and *D. gulyae* varied over among four countries (Table 2.6). *D. helianthi* was isolated at frequencies of 22.2% from samples

received from Yugoslavia and Serbia to 71.4% from Russia and 100.0% from Bulgaria and Croatia (Table 2.6). In contrast, *D. gulyae* was isolated only from samples received from Canada and at a frequency of 35.0% (Table 2.6).

Approximately 600 bp region of the ITS was amplified from 234 and 27 isolates originating from the U.S. and international collaborators respectively, and was used to query the GenBank database directly. However, only 540 bp could be used to compare with the GenBank-retrieved sequences. A BLASTN search of GenBank was performed for the ITS sequences of 163 U.S. isolates and 18 isolates from international collaborators were identified as *D. helianthi* based on comparison with the type isolate from sunflowers (*D. helianthi* strain CBS 592.81, Accession # AY705842) as designated by Muntanola-Cvetkovic *et al.* (1981) (Table 2.5 and Table 2.6). These isolates differed at a single nucleotide site from the type isolate for the ITS gene. A BLASTN search of GenBank was performed for the ITS sequences of the *D. gulyae* U.S. isolates (72 isolates, 30.4%), which showed the best match was *Phomopsis* sp. AJY-2011a strain T12505G (*D. gulyae*, Accession # JF431299) from *H. annuus* in Australia with identities = 540/540(100%) and gaps = 0/540(0%) (Table 2.5 and Table 2.6). A BLASTN search of GenBank performed for the ITS sequences of seven *D. gulyae* isolates from Canada showed the best match was *Phomopsis* sp. AJY-2011a strain T12505G (*D. gulyae*, Accession # JF431299) from *H. annuus* in Australia with identities = 540/540(100%) and gaps = 0/540 (0%) (Table 2.6).

Molecular phylogenetics

For sequences of the 19 U.S. isolates, Bayesian and maximum likelihood analyses produced similar topologies, in analyses of each locus alone (ITS) and combined (EF1α and actin). The ITS alone (Fig. 2.2) and combined data set (Fig. 2.3) were most informative and unknown *Diaporthe* isolates grouped with type specimens into distinct clades.

Of the 19 *Diaporthe* isolates from the U.S., 12 isolates clustered with the type specimen of *D. helianthi* (CBS 592.81, Accession # AY705842) and 7 isolates with the type specimen of *Phomopsis* sp. AJY-2011a strain T12505G (*D. gulyae*, Accession # JF431299) in phylogenetic analyses of the ITS data set (Fig. 2.2). In the ITS tree, Group A represented *D. helianthi* isolates from *H. annuus* in Croatia, France, Yugoslavia and U.S. collection (Fig. 2.2). The separation of these two groups had 90% PP in the Bayesian ITS tree. Group B represented isolates of *D. gulyae* from *H. annuus* in Australia and our collection was placed near to the type sequences of *D. ambigua*, *D. angelicae*, *D. stewartii,* and *D. dauci*, as well as the soybean pathogens *D. longicolla* and *D. phaseolorum*. The *D. gulyae* clade appears as a moderately well supported branch within Group B, with 70% PP, compared to 100% PP for the well-established *D. helianthi* clade (Group A) in the ITS data set (Fig. 2.2).

For further characterization of the U.S. isolates, EF1-α and ACT genes were combined based on the results of the partition-homogeneity test (p = 0.433) indicating that the trees reflect the same underlying phylogeny. Overall there was an increase in bootstrap support when the combined dataset was used (Fig. 2.3). *D. gulyae* formed an exclusive group within the clade (PP=100%), while the soybean pathogens and *Diaporthe* spp. seems to have further evolved from the ancestor forming monophyletic groups supported by high PP within the molecular combined phylogeny (Fig. 2.3).

For characterization of international isolates, a phylogenetic tree was constructed on the basis of the ITS gene sequences using Bayesian analyses resolving the representative 17 *Diaporthe* isolates and types isolates of *D. gulyae* and *D. helianthi* into two coherent clusters (Fig. 2.4). The *D. gulyae* clade appears as a moderately well supported branch, with 70% PP, compared to 100% PP for the well-established *D. helianthi* clade (Fig. 2.4).

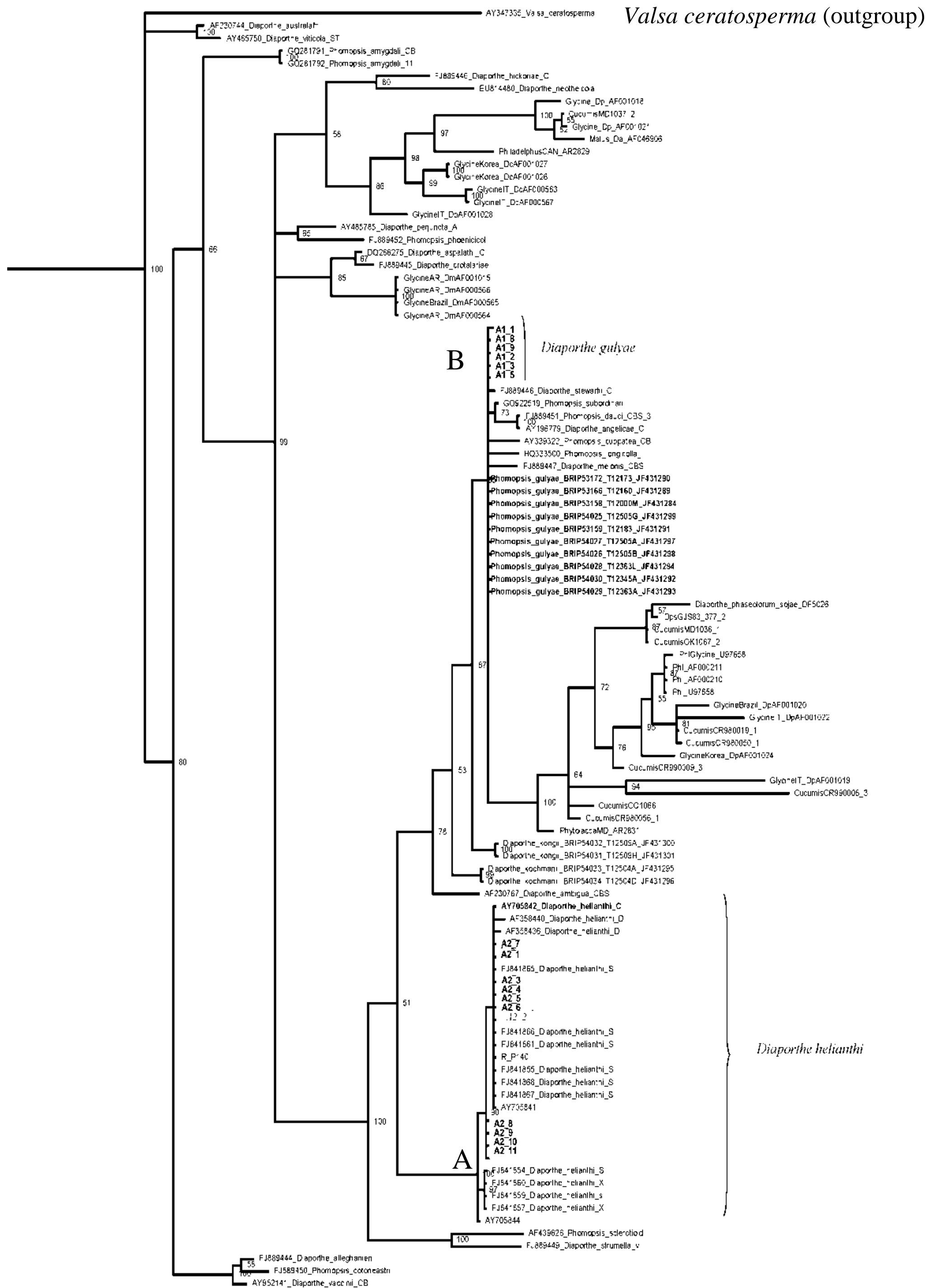

Fig. 2.2. Phylogenetic tree resulting from Bayesian inference analysis of the ITS region of the U.S. *Diaporthe* isolates. Bayesian posterior probabilities (>0.7) for the Bayesian analyses are indicated at the internodes. The tree is rooted with *Valsa ceratosperma* (NCBI Accession number AY347335).

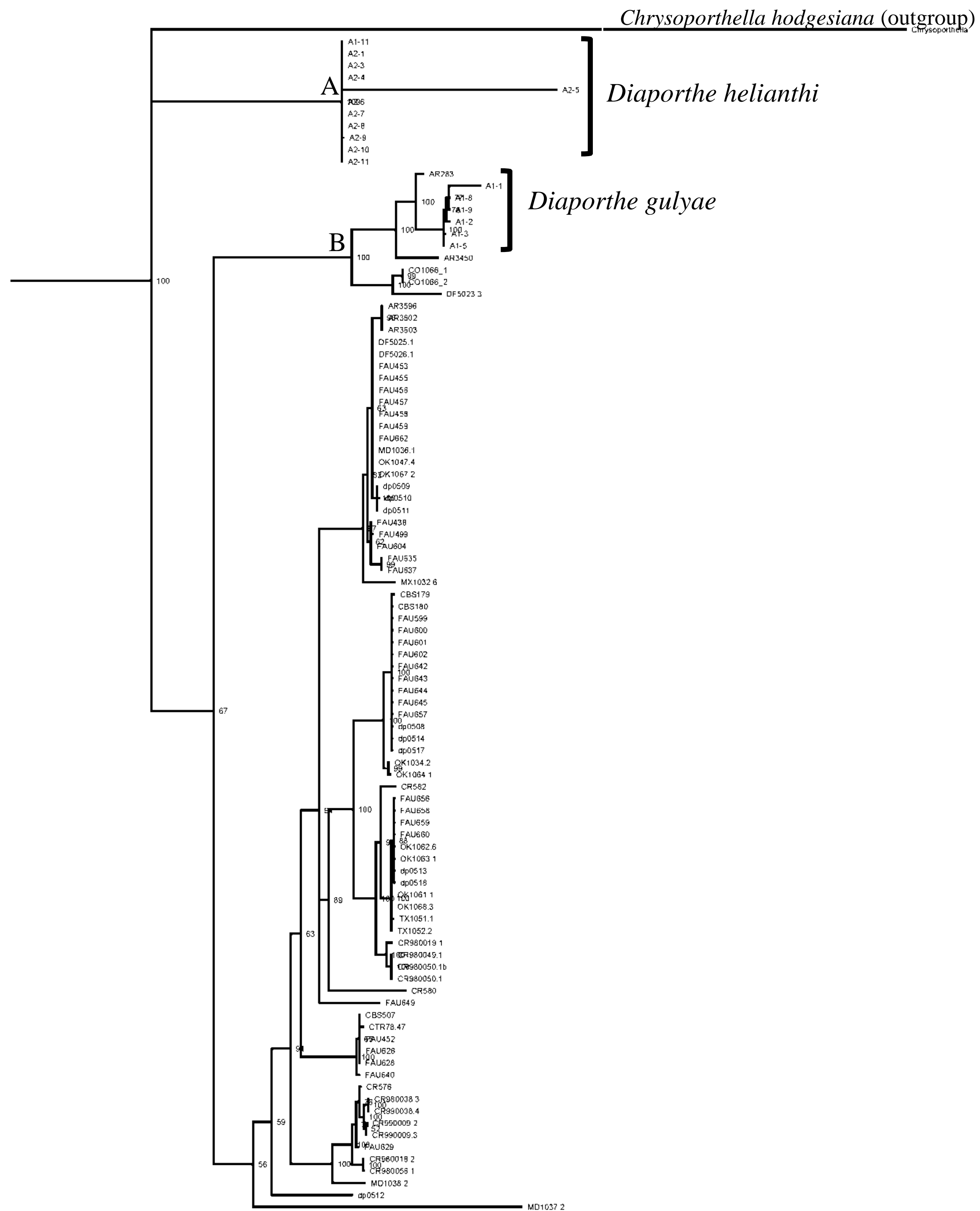

Fig. 2.3. Phylogenetic tree resulting from Bayesian inference analysis of the combined EF1-α and ACT genes region of the U.S. *Diaporthe* isolates. Bayesian posterior probabilities (>0.7) for the Bayesian analyses are indicated at the internodes. The tree is rooted with the outgroup *Chrysoporthella hodgesiana* (NCBI Accession number GQ290152 for EF1-α sequence and GQ290170 for ACT sequence).

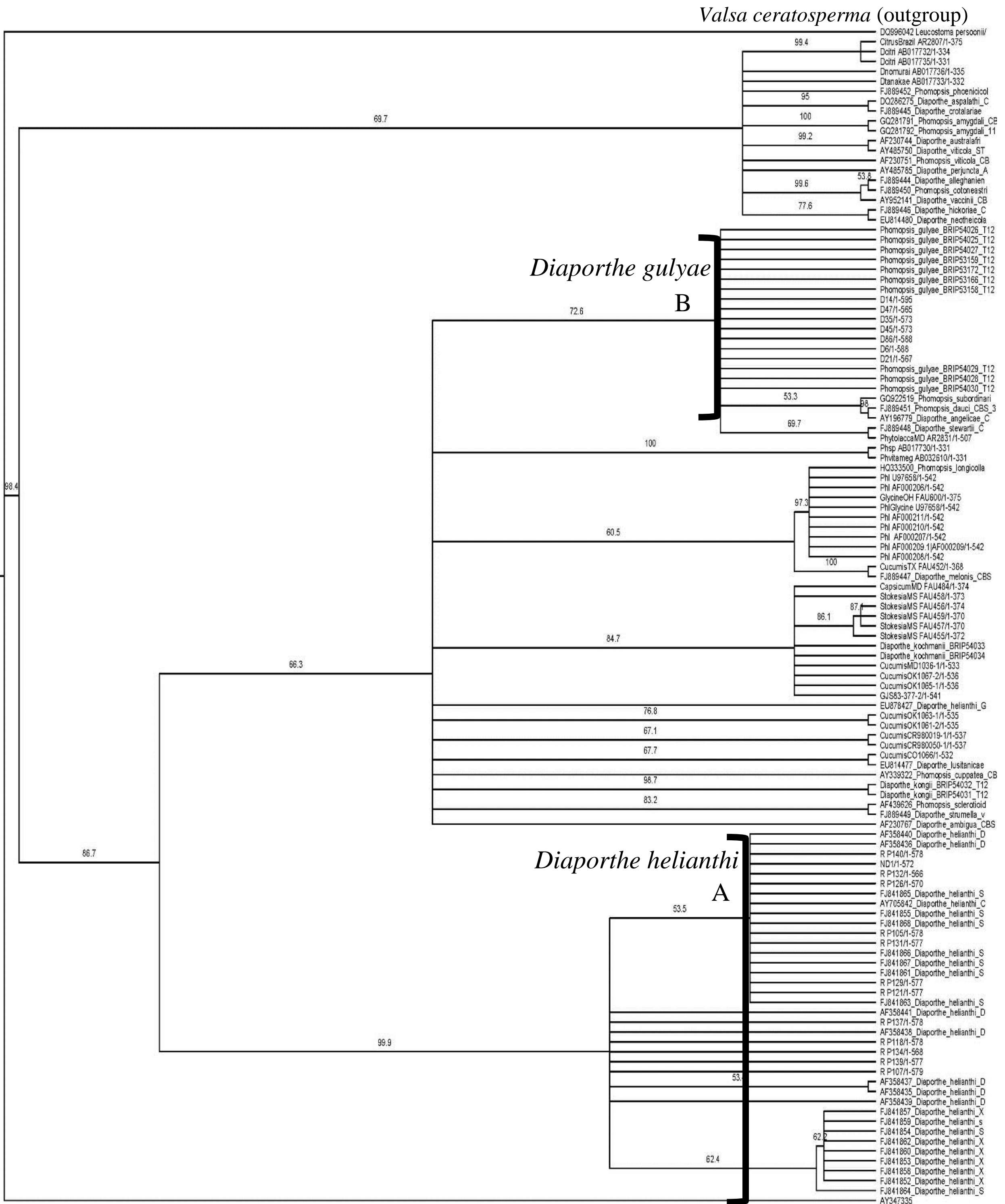

Fig. 2.4. Phylogenetic tree resulting from Bayesian inference analysis of the ITS region of the international *Diaporthe* isolates. Bayesian posterior probabilities (>0.7) for the Bayesian analyses are indicated at the internodes. The tree is rooted with *Valsa ceratosperma* (NCBI Accession number AY347335).

Table 2.7. ***Diaporthe*** **isolates and their mating type PCR reaction**

Isolates[a]	Year of isolation	State	Species identity[b]	Detection of mating type genes[c]	
				MAT1-1-1	MAT 1-2-1
D6	2010	SD	*D. gulyae*	+	+
D9	2010	SD	*D. gulyae*	+	+
D12	2010	SD	*D. gulyae*	+	+
D14	2010	SD	*D. gulyae*	+	+
D21	2010	SD	*D. gulyae*	+	+
D32	2010	SD	*D. gulyae*	+	+
D40	2010	SD	*D. gulyae*	+	+
D25	2010	SD	*D. gulyae*	+	+
D45	2010	SD	*D. gulyae*	+	+
D48	2010	SD	*D. gulyae*	+	+
D86	2010	SD	*D. gulyae*	+	+
D20	2010	SD	*D. gulyae*	+	+
D35	2010	SD	*D. gulyae*	+	+
D47	2010	SD	*D. gulyae*	+	+
R_P131	2010	MN	*D. helianthi*	+	-
R_P129	2010	ND	*D. helianthi*	+	-
R_P132	2011	MN	*D. helianthi*	+	-
R_P126	2011	MN	*D. helianthi*	+	-
R_P121	2011	MN	*D. helianthi*	+	-
R_P105	2011	MN	*D. helianthi*	+	-
R_P140	2011	MN	*D. helianthi*	+	-
R_P137	2011	MN	*D. helianthi*	+	-
R_P134	2012	MN	*D. helianthi*	+	-
R_P118	2012	MN	*D. helianthi*	+	-
R_P139	2012	MN	*D. helianthi*	+	-
R_P107	2012	MN	*D. helianthi*	+	-

[a] Isolates are a subset of 234 isolates, and were chosen as representatives for species-level identification.
[b] Species identity was established based on phylogenetic analysis of the internal transcribed spacer region (ITS), elongation factor subunit 1-α (EF1α) and actin (ACT), conidial dimensions, and colony growth.
[c] Mating type diagnosis was performed using the protocol of Santos *et al.* (2010).

Mating type identification of the U.S. *Diaporthe* isolates

The amplification reactions for MAT1-1-1 and MAT1-2-1 genes were performed, but the sequences were not used in phylogenetic analysis. The mating-type diagnosis using primers showed that all *D. gulyae* isolates in this study study have both MAT1-1-1 and MAT1-2-1 genes suggesting they are homothallic (Table 2.7). For *D. helianthi* isolates, only the MAT1-1 locus was detected (Table 2.7).

Discussion

The results of this study demonstrated that two *Diaporthe* species, *D. helianthi* and *D. gulyae*, are responsible for Phomopsis stem canker on sunflowers in the U.S. The identification of *Diaporthe* species causing Phomopsis stem canker in the U.S. was based on DNA sequence analyses of the ITS, EF-1α and ACT genes for identification of *Diaporthe* species because morphological characteristics are unreliable (van Rensburg *et al*., 2006). Reference isolates of the two *Diaporthe* species were used in the DNA sequence analysis to compensate for the lack of taxonomic characters (Fig. 2.2). Phylogenetic analysis of ITS sequences of *D. helianthi* isolates from the U.S. and international collaborators (Russia, Yugoslavia and Serbia, Bulgaria and Croatia) represent a tight monophyletic clade and revealed that they were closely related to all isolates from France and the former Yugoslavia where losses due to sunflower canker up to 40% (Carre, 1993) and 50% (Pentericci, 1988) have been reported (Figs. 2.2 and 2.4). In contrast, *D. gulyae* isolates from the U.S. and Canada appeared as a moderately well supported clade based on ITS sequences (Figs. 2.2 and 2.4). The consensus BI tree topology inferred from the combined dataset identified two independent lineages for *D. helianthi* and *D. gulyae* supported by higher measures of clade support of 100% PP and 88% PP respectively (Fig. 2.3). To the best of our

knowledge, this is the first report of *D. gulyae* causing Phomopsis stem canker on sunflowers in the U.S. and Canada.

The existence of two *Diaporthe* species on sunflowers in the U.S. could be explained by several hypotheses. Since *D. helianthi* and *D. gulyae* can be transmitted through seed (Herr *et al*., 1983, Thompson *et al*., 2011), one hypothesis would suggest a recent introduction of new species by movement of seed. *Diaporthe helianthi* was confirmed by morphology in Ohio in 1980 (Herr *et al*., 1983), followed by Texas in 1982 (Yang *et al*., 1984), and Minnesota and North Dakota in 1984 (Hajdu *et al*., 1984). We did not have any *Diaporthe* isolates from sunflowers prior to 2010 for use in our molecular study. However, we were able to perform Bayesian analyses of the ITS sequences deposited by Miric (2002) in the GenBank nucleotide database of the two isolates (from ND) collected in 1984 and 1988 (data not presented). The isolates were identified as *D. helianthi* based on comparison with the type isolate strain CBS 592.81 (Accession # AY705842). While this eliminates the hypothesis of a recent introduction of *D. helianthi* into the U.S., we cannot molecularly confirm the same for *D. gulyae*.

Another hypothesis is that both *Diaporthe* species were endemic in the U.S. Herr *et al*. (1983) reported that *Diaporthe* isolates identified causing Phomopsis stem canker in Ohio produced pycnidia with predominantly α-conidia, some with predominantly β-conidia or those with both the conidia types. They concluded that the *Diaporthe* isolates that produced predominantly β-conidia were similar to *D. helianthi*, described in Yugoslavia. However, they were not able to resolve inconsistencies in the description of the *Diaporthe* isolates found in Ohio in comparison to those from Yugoslavia (Mihaljcevic *et al*., 1985), particularly for those that produced predominantly α-conidia. In our study, *D. helianthi* isolates produced white, floccose, and dense with dark brown colored mycelia on PDA; size of β-conidia ($20 – 35 \times 0.5 – 2.7$ μm)

was consistent with Gulya *et al*. (1997). In contrast, *D. gulyae* appeared flat in the center of the PDA plate and irregularly fluffy near the margin with few aerial hyphae, and the size of α-conidia (6.5 – 8.5 × 2.5 – 3.5 μm) was fairly consistent with Thompson *et al*. (2011). We did not observe β-conidia in *D. gulyae*. Additionally, *D. gulyae* caused leaf wilting, necrosis and lodging in our aggressiveness study ((Mathew *et al*., unpublished [chapter 3]); these matched the symptoms described by Herr *et al*. (1983) but not those described by Mihaljcevic *et al*. (1985) for *D. helianthi*. Comparing the biological differences among *Diaporthe* isolates described by the previous researchers and in our study, it is likely that the two *Diaporthe* species were already present in the U.S. at low levels, but the ability to distinguish them prior to molecular techniques did not exist.

In the present study, mating type primers designed by Santos *et al.* (2010) was used to determine if the two *Diaporthe* species are homothallic or heterothallic. Since both mating-type loci are present in the genome of *D. gulyae* isolates, the pathogen can be regarded homothallic. In contrast, only MAT1-1 locus was detected for *D. helianthi* isolates, the results of which was consistent with that of Santos *et al*. (2010) and Udayanga *et al*. (2012). However, mating tests using the method of Brayford (1990) suggests that *D. helianthi* is homothallic (Santos, personal communication). As described by Santos *et al.* (2010), the inability to detect MAT1-1-1 in *D. helianthi* isolates in this study could be due to point mutations in the annealing regions of the primers and further analyses of mating-type genes was beyond the scope of our study.

The present study does suggest that *D. helianthi* was more prevalent than *D. gulyae* in the Northern Great Plains and they were not distributed in equal amounts across years or states. State and year variation between occurrences of *Diaporthe* species is likely a result of differences in sampling strategies as opposed to actual distribution of the two species. For instance, in 2010, sampling of stalks was based on the appearance of any stem disease and associated symptoms such

as wilting, lodging and plant death that compromised sunflower yield. In 2011 and 2012, sunflower stalks were sampled from fields heavily infected by Phomopsis stem canker. In these fields *D. helianthi* was most frequently identified, which suggests *D. helianthi* may be more of an economic concern for sunflower production in the Northern Great Plains than *D. gulyae*. Year-to-year variation between species occurrence has been documented previously in pathogens such as *Fusarium* and this was attributed to inoculum source and overwintering structures (Backhouse and Burgess, 2002). For *Diaporthe* spp., although the perithecium is known to survive in debris for five years (Herr *et al*., 1983), there are no studies evaluating the influence of environmental conditions and perithecia survival on the ability of the fungus to develop and persist as a pathogen. Although it is likely that environmental conditions or perithecia survival may have an influence on the distribution of the two *Diaporthe* spp. in the Northern Great Plains, this was beyond the scope of our study.

This study began in response to an increase in incidence, severity and reports of yield losses to Phomopsis stem canker in the Northern Great Plains. While it is possible that changes in the *Diaporthe* populations in the U.S. have contributed to this, our results suggest it is more likely that an increase in disease is a result of other factors, and that the simultaneous identification of *D. gulyae* is circumstantial. One reason for increased Phomopsis stem canker may be the increased production of sunflowers on no-till and minimum tillage field ground which would contribute to an increase in inoculum. Additionally, awareness of Phomopsis stem canker has increased in the last five years. As this disease is relatively difficult to distinguish from Phoma black stem (caused by *Phoma macdonaldii* Boerema), another common stem disease in the Northern Great Plains (Kandel, 2012), it is possible that some of the increased incidence of Phomopsis stem canker may be an artificial response due to an increased awareness campaign and subsequent misidentification.

In this study, we were able to identify *Diaporthe* species prevalent in major sunflower production regions of the world and compare with those in the U.S. (for example, *D. helianthi* from Russia and *D. gulyae* from Canada). However, one limitation in that comparison is the sample size of the international isolates. In sunflower samples obtained from international collaborators, we recovered isolates of *Verticillium* spp. and *Fusarium* spp. rather than *Diaporthe* spp. For instance, these two pathogens were recovered from stems that had stem canker-like symptoms received from China and Turkey. This may be because Phomopsis stem canker was misdiagnosed by the surveyors or collectors, given similarities between symptoms of stem canker and other late season diseases or mixed infections with other pathogens such as Phoma black stem, Verticillium wilt (*Verticillium dahliae* Kleb.), Fusarium wilt (*Fusarium* spp.) and charcoal rot (*Macrophomina phaseolina* (Tassi) Goid). Due to the economic importance of Phomopsis stem canker worldwide, an international study of distribution of *Diaporthe* species may be warranted. Sunflower production is international in nature and the same cultivars are grown and bred in multiple countries. Consequently, identification of the international pathogen populations may benefit breeding for resistance and management of Phomopsis stem canker in the future. In addition, correct identification of *Diaporthe* species on sunflowers is essential for international phytosanitary measures, particularly because *D. helianthi* is a quarantined pathogen in most countries (USDA-Foreign Agricultural Service, 2005-2011).

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CHAPTER TWO. PATHOGENICITY OF *DIAPORTHE* SPECIES INFECTING SUNFLOWERS IN THE UNITED STATES

Abstract

Phomopsis stem canker is an economically important disease on sunflowers (*Helianthus annuus* L.). In the United States (U.S.), according to the annual survey coordinated by the National Sunflower Association (NSA), disease incidence has increased from ~ 1.5% of the crop in 2001 to 24.4% in 2012. Historically, *Diaporthe helianthi* M. Muntanola-Cvetkovic *et al.* was assumed to be the sole causal agent of the disease. However, *D. gulyae* Shivas, Thompson and Young, was recently identified in Australia and in 2010, was found to be widespread in the Northern Great Plains in the U.S. The objectives of this study were (a) to evaluate greenhouse inoculation methods for *D. helianthi* on sunflower, (b) to compare the aggressiveness of *D. helianthi* and *D. gulyae*, and (c) to evaluate sunflower accessions for resistance to the two *Diaporthe* spp. Four methods were tested to assess the Phomopsis stem canker response using four isolates of *D. helianthi* on a susceptible sunflower plant. The four methods were stem-wound, wound-inoculation, petiole-wound and straw test. The stems of the plants were inoculated with mycelial plugs by wounding the stem with micropipette tips or sliting the stem for the stem-wound and wound-inoculation methods respectively. For petiole- wound experiments, wounding was made with a micropipette tip (100 µl) on petiole and the mycelial plug was placed over the wounded petiole. For the straw test, sunflowers plants were inoculated by cutting the leaf petiole and placing a micropipette tip containing the mycelial plug of *D. helianthi* isolates. All plugs were attached to the wounds with Parafilm to avoid a rapid dehydration. Although infection was successful with all the *D. helianthi* isolates when different inoculation methods were used, recovery of the pathogen differed significantly ($p<0.0001$) among methods. Based on a higher mean recovery of *D. helianthi* isolates

from the inoculated plants, the stem-wound method was adopted to compare aggressiveness between species and among isolates within the two *Diaporthe* spp. The results of this study demonstrated that *D. helianthi* and *D. gulyae* did not vary significantly (p=0.0012) in their aggressiveness; however the isolates within the two species varied. Among the nine genotypes screened for Phomopsis stem canker resistance using stem-wound method, USDA 'PI 162784' and USDA 'PI 219649' were relatively less susceptible to *D. helianthi* and *D. gulyae* based on stem inoculations.

Introduction

Phomopsis stem canker in the United States is caused by two species of *Diaporthe* (Mathew *et al*., unpublished [chapter 2]), namely, *Diaporthe helianthi* M. Muntanola-Cvetkovic *et al.* (Muntañola-Cvetković *et al.*, 1985) and *Diaporthe gulyae* Shivas, Thompson and Young (Thompson *et al.*, 2011). Historically, the disease has been one of the primary limiting factors for sunflower production in Europe, where yield losses up to 50% and losses in oil content in excess of 10% on sunflowers (*Helianthus annuus* L.) have occurred (Masirevic and Gulya, 1992) but had only a limited impact in the United States. However, incidence of fields with the disease in the United States has increased from approximately 1.5% in 2001 to 24.4% in 2012 (Kandel, 2012). Concurrently, 50% yield reductions due to Phomopsis stem canker have been reported from commercial fields and seed nurseries in Northern Minnesota and North Dakota between 2010 and 2012 (Markell and Gulya, personal communication). In response, the National Sunflower Association (NSA) in the United States is researching into developing management tools for Phomopsis stem canker, which is one of their top research priorities since 2010.

The term "aggressiveness", as used in this chapter, is based on the quantitative variation of pathogenicity on susceptible hosts (Pariaud *et al*., 2009). Understanding the aggressiveness of the

Diaporthe species in the United States is important for developing management strategies, particularly as it relates to breeding for resistance. Variation in aggressiveness among *D. helianthi* isolates has been reported on sunflowers (Entcheva, 2002). Herr *et al*. (1983) examined the reaction of 15 *D. helianthi* isolates inoculated to commercial sunflower hybrids and observed variation in aggressiveness characteristics such as lesion size and infectious period. In addition, the severity of symptoms expressed in these commercial sunflower hybrids inoculated with the same isolate varied from plant to plant complicating phenotyping (Herr *et al*., 1983). Variation in symptoms and lesion size among *D. gulyae* isolates was reported on sunflowers by Thompson *et al*. (2011). Says-Lesage *et al*. (2002) further showed that extensive genetic differences occur between *D. helianthi* strains from different geographical origins, so it is possible that there may be genetic variability between the populations of *D. helianthi* and *D. gulyae* occurring in the U.S. and in other sunflower production regions of the world, making evaluation of local populations critical for disease management. Additionally, *D. helianthi* has caused consistent and significant yield losses in Europe (Masirevic and Gulya, 1992). Similarly, Thompson *et al*. (2011) reported *D. gulyae* caused severe Phomopsis stem canker on sunflower in Australia. However, there are no studies making a direct comparison of aggressiveness and yield impact of *D. gulyae* and *D. helianthi*.

Genetic resistance is commonly the most cost effective and environmentally friendly way to manage diseases. The majority of resistance breeding efforts for Phomopsis stem canker have been under natural-infection conditions in the field. Natural infection has provided good discrimination among genotypes, but only in years and in environments with high infection pressure (Degener *et al*., 1999). Additionally, Langer *et al*. (2000) found that the resistance to *D. helianthi* in sunflower germplasm was distributed relatively evenly with a range from highly

susceptible to highly resistant under natural-infection conditions. However, selection of resistant hybrids in the field takes an entire growing season to complete, and results are heavily dependent on environmental conditions. Screening for resistance in greenhouse conditions would be advantageous, but has been challenging. While ascospores are widely regarded as the primary source of inoculum, the *in vitro* production of them is difficult and has limited the ability of pathologists to screen host germplasm under inoculation conditions that best mimic the disease cycle (Viguié *et al*., 1999).

Several artificial inoculation methods have been developed for inoculating *Diaporthe* spp. on sunflowers under controlled conditions and assessing aggressiveness of the pathogen (Thompson *et al*., 2011, Encheva and Kiryakov, 2002, Tourvieille *et al*., 1988). All of these inoculation methods used mycelial plug as the source of inoculum. For example, Encheva and Kiryakov (2002) investigated the use of straw method using mycelial plug as the inoculum for testing sunflower response to *D. helianthi*. Briefly, plants were inoculated at vegetative stage by cutting the leaf petiole by a scalpel and inserting a plastic straw containing an agar plug of 7-d old *D. helianthi* culture. A significant correlation was observed between disease severity from the straw test using mycelial plug as the source of inoculum and disease severity from natural infection by ascospores (Encheva and Kiryakov, 2002). Thompson *et al*. (2011) used two evaluation methods (wound inoculation and mycelium contact method) to quantify the severity of Phomopsis stem canker. Briefly, for the wound-inoculation method, plants were inoculated at V6-V8 stage either by making a long slit in the stem with a scalpel and placing an agar plug of 7-d old *Diaporthe* spp. culture into the slit. The mycelium contact method used by Thompson *et al*. (2011) was less invasive and it involved placing an inoculated agar plug in contact with the stem at a node. A visual rating on a 0-to-5 scale was developed by Thompson *et al*. (2011) with lesion length being

estimated based on discoloration at the site of inoculation, stem streaking, wilting, lodging or plant death.

In spite of the available inoculation methods in Phomopsis stem canker studies carried out under controlled conditions, qualitative comparisons of these methods are lacking. The objectives of this study were (a) to evaluate greenhouse inoculation methods for *D. helianthi* on sunflower, (b) to compare the aggressiveness of *D. helianthi* and *D. gulyae*, and (c) to evaluate sunflower accessions for resistance to the two *Diaporthe* spp.

Materials and method

Comparison of inoculation methods

To determine an effective greenhouse-based inoculation technique, four inoculation methods; stem-wound, petiole-wound, wound-inoculation (Thompson *et al*., 2011) and straw test (Encheva and Kiryaakov, 2002) were evaluated.

The trial was conducted using the USDA confection inbred line 'HA 288' which is considered universally susceptible to *D. helianthi*. Two seeds were planted in moist planting mix (Sunshine Mix # 1, Sun Grow Horticulture Products, Belleview, WA) in 7.5 liter circular plastic pots. The pots were placed on the greenhouse benches at 22 to 25°C under a 16-h light/dark cycle and watered on alternate days.

Four isolates of *D. helianthi* were selected to test the inoculation methods based on preliminary aggressiveness greenhouse studies (Table 3.2). These included two isolates from Minnesota (2011), one isolate from North Dakota (2010) and one isolate from South Dakota (2011). The *D. helianthi* isolates were cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 0.02% streptomycin sulfate. Plates were incubated at room temperature for 10-d under 12-h of alternating light and dark conditions. Mycelial plugs (4 mm in diameter)

were taken from the margin of the growing colony and used as inoculum for all inoculation methods tested.

For the wound-inoculation method, inoculation points were made on each stem by making a vertical slit on the 2nd internode of approximately 5 mm in length using a sterile scalpel (Thompson *et al*., 2011). A mycelial plug (4 mm diameter) from the margin of a 10-d PDA culture of the four *D. helianthi* isolates was placed into the slit made in the stem and wrapped with Parafilm to avoid a rapid dehydration. Control plants were wounded with a 5 mm long slit at the nodes and non-infested PDA plug placed in the slit.

For the stem- and petiole- wound experiments, wounding was made with a micropipette tip (100 µl) on the 2nd internode and petiole, respectively. A mycelial plug (4 mm diameter) from the margin of a 10-d PDA culture of the four *D. helianthi* isolates was placed over the wounded stem/petiole. Control plants were wounded with micropipette tips on the stem/ petiole and non-infested PDA plug placed on the wound. All plugs were attached to the wounds with Parafilm to avoid a rapid dehydration.

For the straw test (Encheva and Kiryakov, 2002), sunflowers plants were inoculated by cutting the leaf petiole at an approximate distance of 3 cm from the stem by a scalpel. A micropipette tip containing an agar plug of 10-d old culture of the four *D. helianthi* isolates was inserted into the core of the cut sunflower petiole. Micropipette tips containing plugs of non-inoculated PDA served as the control. All plugs were attached to the wounds with Parafilm to avoid rapid dehydration.

Disease severity was evaluated at 14-d after inoculation using a 0 to 5 scale modified from Thompson *et al*. (2011), where; 0 = no discoloration; 1 = low level discoloration at site of inoculation; 2 = slight discoloration or lesion 1–2 mm in length; 3 = necrotic lesions 2–5 mm in

length, some colored stem streaking, leaf wilting and twisting; 4 = lesions 5–10 mm in length, significant necrosis and dark colored stem streaking, leaf and plant wilting, twisting, stunting, and some lodging; 5 = lesions exceeding 10 mm in length, severe leaf necrosis, lodging or plant death.

The assessment of inoculation methods was further made based on the recovery of *D. helianthi* from the inoculated plants. Plants were harvested 14-d after inoculation and pieces (approximately 1 cm length) were sectioned longitudinally from the point of inoculation of the infected tissue. The pieces were washed with tap water for 2 min, surface-sterilized in sodium hypochlorite (10%) and ethanol (70%) for 1 min each, rinsed in sterile distilled water four times and blotted between sterile filter papers. Four pieces were placed on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 0.02% streptomycin sulfate. Plates were incubated at room temperature for 7- to 14-d under 12-h of alternating light and dark conditions. Cultures were scored for presence or absence of *D. helianthi* based on morphology (Barnett and Hunter, 1972). Association between disease severity at 14-d for different inoculation methods and recovery of *D. helianthi* was quantified with Spearman rank correlation coefficients (Spearman, 1904) using the PROC CORR procedure on SAS v9.3 (SAS Institute, Cary, NC).

A factorial experiment arranged in a completely randomized design with combinations of four inoculation methods and four *D. helianthi* isolates was established and the experiment was repeated twice. The experimental unit is the single plant in each pot and each treatment was replicated 12 times (12 plants) in each combination of inoculation method and isolate. Analysis of data based on observed ranks was performed in SAS. The ordinal data from the disease rating scale did not have a normal distribution; therefore they were analyzed using the nonparametric procedure of Brunner *et al*. (2002) as described by Shah and Madden (2004). Preliminary data analysis showed that the results of both experimental runs were very similar, and so the data from

both runs were combined for the final analysis. The overall and interaction effect of inoculation methods and *D. helianthi* isolates on disease severity was determined by the analysis of variance type statistic (ATS) of ranked data (Singer *et al*., 2004). Interaction effects that the effect of the ith level of A on the distribution depends on the level of B, or that the effect of the jth level of B depends on the level of A, where A and B are two experimental factors. The ATS has an approximate F distribution (with df_N [numerator] and df_D [denominator] degrees of freedom) under the null hypothesis. The ATS has an approximate F distribution (with df_N [numerator] and df_D [denominator] degrees of freedom) under the null hypothesis. The PROC RANK procedure in SAS was used to obtain midranks followed by PROC MIXED to generate relative effects (REs). The null hypothesis (H_o) is that the RE of all treatments are the same, while the alternative hypothesis (H_A) is that at least one of the relative treatment effects (RE) is different from the rest from the other treatments. RE, defined by means of probabilities derived from the marginal distribution functions of disease severity, are generated by the equation: $RE = (R - 0.5)/N$; where R is the mean treatment ranking and N is the total number of observations in the data analysis. Confidence intervals were calculated using LD_CI macro in SAS at $p \leq 0.05$ (Brunner *et al*., 2002).

Aggressiveness of *Diaporthe* species

The stem-wound method was adopted to compare aggressiveness between *Diaporthe* species and among isolates within the two species based on the results of the previous experiment. Ten isolates of *D. helianthi* and *D. gulyae* were randomly selected from collections made during a stem-disease survey in the North Great Plains between 2010 and 2012 (Mathew *et al*., unpublished [chapter 2]) (Table 3.4). These included ten *D. gulyae* isolates from SD, eight *D. helianthi* isolates from Minnesota, one isolate of *D. helianthi* from North Dakota and one isolate of *D. helianthi* from South Dakota. Isolates of *Diaporthe* spp. were cultured on PDA amended with 0.02%

streptomycin sulfate. Plates were incubated at room temperature for 10-d under 12-h of alternating light and dark conditions. Mycelial plugs (4 mm in diameter) were taken from the margin of the growing colony and used as inoculum to compare the aggressiveness of the two *Diaporthe* species.

The stems of sunflower plants 'HA 288' between the V4 and V6 growth stages were wounded on the 2nd internode and a *Diaporthe*-infested mycelial plug placed on the wound. The pots were placed on the greenhouse benches at 22 to 25°C under a 16-h light/dark cycle and watered alternate days. Because differences in disease symptoms were observed between the two *Diaporthe* species at 3-d, 10-d and 14-d after inoculations, repeated disease assessments were performed at these times on the same experimental unit (sunflower plant). Plants were assessed for lesion development on a scale of 0 to 5 as described previously (Thompson *et al*., 2011).

The trial was conducted in a completely randomized design with 12 plants evaluated per treatment and the experiment was repeated twice. The experimental unit is the single plant in each pot and each treatment was replicated 12 times (12 plants). Analysis of data based on observed ranks was performed in SAS v9.3 (SAS Institute, Cary, NC). The ordinal data from the disease rating scale did not have a normal distribution; therefore they were analyzed using the nonparametric procedure of Brunner *et al*. (2002) as described by Shah and Madden (2004). Preliminary data analysis showed that the results of both experimental runs were very similar, and so the data from both runs were combined for the final analysis. A repeated-measures analysis was used to identify significant effects of time on the disease produced by the two *Diaporthe* species and isolates within species. The overall and interaction effect of *Diaporthe* species and time on disease severity was determined by the analysis of variance type statistic (ATS) of ranked data (Singer *et al*., 2004). The overall and interaction effect of *Diaporthe* isolates within species and time on disease severity was also determined by the ATS of ranked data. The ATS has an

approximate F distribution (with df_N [numerator] and df_D [denominator] degrees of freedom) under the null hypothesis. However, corrections to the calculated df_D were made in order to obtain $df_D = \infty$ for the tests of time and and the interaction of *Diaporthe* species with time as explained by Shah and Madden (2004) and Brunner *et al*. (2002). The PROC RANK procedure in SAS was used to obtain midranks followed by PROC MIXED to generate relative effects (REs). The null hypothesis (H_o) is that the RE of all treatments are the same, while the alternative hypothesis (H_A) is that at least one of the relative treatment effects (RE) is different from the rest from the other treatments. RE, defined by means of probabilities derived from the marginal distribution functions of disease severity, are generated by the equation: $RE = (R - 0.5)/N$; where R is the mean treatment ranking and N is the total number of observations in the data analysis. Confidence intervals were calculated using LD_CI macro in SAS at $p \leq 0.05$ (Brunner *et al*., 2002).

In addition to the comparing aggressiveness the two *Diaporthe* species and isolates within species, the experiment was also used to complete Koch's postulates of *D. gulyae*, which was not previously reported as a pathogen of sunflowers causing Phomopsis stem canker in the U.S.

Screening sunflower genotypes for resistance to the two *Diaporthe* species

To evaluate potential differences in genetic resistance to the two *Diaporthe* species, nine USDA plant introductions (PI) that exhibited resistance to Phomopsis stem canker in a recent field study by Talukder *et al*. (2014) were obtained. Seeds of the nine PIs included were obtained from the USDA North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA. They were from Argentina, Hungary, Czechoslovakia, Austria, Canada, Poland, China, Yugoslavia and Serbia (Table 3.7). The USDA confection inbred 'HA 288' was used as the susceptible check. All genotypes were grown under greenhouse conditions at 22 to 25°C under a 16-h light/dark cycle and watered alternate days. The stem-wound method using mycelial plug (4 mm in diameter) as

the source of inoculum was adopted for this study. Plants were inoculated at V4 and V6 growth stages by wounding on the 2nd internode and inoculated with the most aggressive isolate of *D. helianthi* (DH8) and *D. gulyae* (DG8) found in the previous experiment. Plants were assessed for lesion development at 10-d after inoculation on a scale of 0 to 5 (modified from Thompson *et al*., 2011) based on the results of the previous experiment.

The experiment was designed as a completely randomized design with 12 plants (V4-V6 stage) evaluated per treatment and the experiment was repeated twice. The experimental unit is the single plant in each pot and each treatment was replicated 12 times (12 plants). Analysis of data based on observed ranks was performed in SAS v9.3 (SAS Institute, Cary, NC). The ordinal data from the disease rating scale did not have a normal distribution; therefore they were analyzed using the nonparametric procedure of Brunner *et al*. (2002) as described by Shah and Madden (2004). Preliminary data analysis showed that the results of both experimental runs were very similar, and so the data from both runs were combined for the final analysis. The overall effect of *Diaporthe* species on disease severity of the sunflower genotypes was determined by the analysis of variance type statistic (ATS) of ranked data (Singer *et al*., 2004). The ATS has an approximate F distribution (with df_N [numerator] and df_D [denominator] degrees of freedom) under the null hypothesis. The PROC RANK procedure in SAS was used to obtain midranks followed by PROC MIXED to generate relative effects (REs). The null hypothesis (H_o) is that the RE of all treatments are the same, while the alternative hypothesis (H_A) is that at least one of the relative treatment effects (RE) is different from the rest from the other treatments. RE, defined by means of probabilities derived from the marginal distribution functions of disease severity, are generated by the equation: RE = (R – 0.5)/N; where R is the mean treatment ranking and N is the total number

of observations in the data analysis. Confidence intervals were calculated using LD_CI macro in SAS at $p \leq 0.05$ (Brunner *et al*., 2002).

Results

Comparison of inoculation methods

Test statistics indicated no significant effect of experiment or interaction effect between experiment and inoculation methods or experiment and *D. helianthi* isolates or interactions between experiment, inoculation methods and *D. helianthi* isolates in the overall development of Phomopsis stem canker on sunflowers (data not presented). A significant interaction was observed between inoculation methods and *D. helianthi* isolates ($p < 0.0001$) indicating that the inoculation methods influenced the amount of disease caused by the *D. helianthi* isolates 14-d after inoculation (Table 3.1).

Table 3.1. Test statistics for the effects of inoculation method and *D. helianthi* isolates on Phomopsis stem canker of sunflower

	ANOVA type statistic (ATS)			
Source of variation	df_N (numerator degrees of freedom)	df_D (denominator degrees of freedom)	F	*p*-value
Method	2.2	53.7	108.34	<0.0001
Isolates	2.92	53.7	99.35	<0.0001
Method * Isolates	4.35	53.7	38.63	<0.0001

Disease caused by four *D. helianthi* isolates was evaluated using RE and their 95% CI was evaluated at 14-d after inoculation using RE and their 95% CI (Table 3.2). The interaction was because the four *D. helianthi* isolates differed in their RE when the petiole-wound was used as compared to when the other inoculation methods were used (Table 3.2). For example, the RE of DH1 was lower and not significantly different from the control, when the petiole method was used. In contrast, RE of DH1 was higher when the other three inoculation methods were used (Table 3.2). Similarly, while the RE of DH2 was higher when the petiole method was used, it was lower

when the stem-wound and wound-inoculation methods were used (Table 3.2). In general, the RE of the four *D. helianthi* isolates was highest when straw-method was used for inoculations (Table 3.2). The RE of the four *D. helianthi* isolates was not significantly different from each other when the stem-wound and wound-inoculation methods were used (Table 3.2).

Table 3.2. Median, rank, and relative treatment effects for Phomopsis stem canker severity on sunflower caused by *D. helianthi* isolates using different inoculation methods

Inoculation Method	*D. helianthi* isolate	Location, Year	Median disease rating[a]	Mean rank	Estimated relative effect (RE)[b]	Confidence interval (95%) for relative treatment effect	
						Lower limit	Upper limit
Petiole-wound	DH1	MN, 2011	2.0	85.0	0.35	0.34	0.37
	DH2	ND, 2010	2.5	139.0	0.58	0.45	0.69
	DH3	SD, 2011	3.0	193.0	0.80	0.79	0.81
	DH4	MN, 2011	3.0	193.0	0.80	0.79	0.81
	Control		2.0	85.0	0.35	0.34	0.37
Straw test	DH1	MN, 2011	3.0	193.0	0.80	0.79	0.81
	DH2	ND, 2010	3.0	193.0	0.80	0.79	0.81
	DH3	SD, 2011	2.5	139.0	0.58	0.45	0.69
	DH4	MN, 2011	3.0	184.0	0.76	0.69	0.83
	Control		1.0	36.7	0.15	0.09	0.25

[a] Disease severity ratings was evaluated at 14-d after inoculation using a 0 to 5 scale modified from Thompson *et al*. (2011), where; 0 = no discoloration; 1 = low level discoloration at site of inoculation; 2 = slight discoloration or lesion 1–2 mm in length; 3 = necrotic lesions 2–5 mm in length, some colored stem streaking, leaf wilting and twisting; 4 = lesions 5–10 mm in length, significant necrosis and dark colored stem streaking, leaf and plant wilting, twisting, stunting, and some lodging; 5 = lesions exceeding 10 mm in length, severe leaf necrosis, lodging or plant death.
[b] Relative treatment effects were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004).

Table 3.2. Median, rank, and relative treatment effects for Phomopsis stem canker severity on sunflower caused by *D. helianthi* isolates using different inoculation methods (contd.)

Inoculation Method	*D. helianthi* isolate	Location ,Year	Median disease rating[a]	Mean rank	Estimated relative effect (RE)[b]	Confidence interval (95%) for relative treatment effect	
						Lower limit	Upper limit
Wound-inoculation	DH1	MN, 2011	3.0	193.0	0.80	0.79	0.81
	DH2	ND, 2010	2.0	85.0	0.35	0.34	0.37
	DH3	SD, 2011	2.0	85.0	0.35	0.34	0.37
	DH4	MN, 2011	2.0	85.0	0.35	0.34	0.37
	Control		1.0	36.6	0.15	0.09	0.25
Stem-wound	DH1	MN, 2011	3.0	193.0	0.80	0.79	0.81
	DH2	ND, 2010	2.0	85.0	0.35	0.34	0.37
	DH3	SD, 2011	2.0	85.0	0.35	0.34	0.37
	DH4	MN, 2011	2.0	85.0	0.35	0.34	0.37
	Control		1.0	36.7	0.15	0.09	0.25

[a] Disease severity ratings was evaluated at 14-d after inoculation using a 0 to 5 scale modified from Thompson *et al*. (2011), where; 0 = no discoloration; 1 = low level discoloration at site of inoculation; 2 = slight discoloration or lesion 1–2 mm in length; 3 = necrotic lesions 2–5 mm in length, some colored stem streaking, leaf wilting and twisting; 4 = lesions 5–10 mm in length, significant necrosis and dark colored stem streaking, leaf and plant wilting, twisting, stunting, and some lodging; 5 = lesions exceeding 10 mm in length, severe leaf necrosis, lodging or plant death.
[b] Relative treatment effects were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004).

The four *D. helianthi* isolates produced typical Phomopsis stem canker symptoms including elongated lesions and pith discoloration on the stems/petioles when different inoculation methods were used. For example, the infected plants had elongated brown to reddish brown lesions on stalks with a range of 12.7 to 20 mm in length for stem-wound and from 12.7 to 45.7 mm in length for wound-inoculation at 14-d after inoculation. On plants inoculated using the petiole-

wound and straw methods, lesions reached the main stem from the petiole and caused wilting when the lesions expanded transversely towards the main stem. The lesion length ranged from 2.54 to 12.7 mm when the petiole-wound was used and from 6.3 to 12.7 mm when the straw test was used.

To make a qualitative comparison of the four inoculation methods, *D. helianthi* was recovered and averaged across four isolates for each method. The mean recovery of *D. helianthi* (in percentage) differed significantly ($p < 0.0001$) among the inoculation methods (Table 3.3). The lowest re-isolation percentage was obtained from plants inoculated with the straw test (33.3%) and petiole method (31.7%); while the highest percentage was obtained from inoculated plants exposed to stem-wound method (73.3%) (Table 3.3). No pathogen isolated from any control plant (data not presented).

Table 3.3. Mean recovery of *D. helianthi* isolates from sunflower plants inoculated using different inoculation methods

Method	Mean recovery of *D. helianthi* (%)
Stem-wound	73.3 a
Wound-inoculation	68.3 a
Petiole-wound	31.7 b
Straw test	33.3 b
LSD ($p \leq 0.05$)	18.17
p-value	< 0.0001
Coefficient of variation (CV)	74.43

The Spearman rank correlations between disease severity at 14-d after inoculation and recovery of *D. helianthi* was moderate and highly significant ($p < 0.0001$) for stem-wound (ρ = 0.52) and wound inoculation ($\rho = 0.51$). For petiole-wound method, the rank correlation coefficient was very low ($\rho = 0.11$) and not significant at $p = 0.37$. For straw test, the rank correlation coefficient was low ($\rho = 0.30$) and significant at $p = 0.02$. Based on the recovery of *D. helianthi*

and its correlation with disease severity at 14-d after inoculation, stem-wound method was adopted for subsequent experiments.

Aggressiveness of *Diaporthe* species

Test statistics indicated no significant effect of experiment or interaction effects between experiment and *Diaporthe* species or isolates with species in the overall development of Phomopsis stem canker on sunflowers (data not presented).

The repeated measures analysis identified a significant interaction was between *Diaporthe* species and time at p = 0.0009 (Table 3.4). Disease caused by the two *Diaporthe* species and their isolates was evaluated at 3-d, 10-d and 14-d after inoculations using RE and their 95% CI (Table 3.5). The interaction was because *D. gulyae* and *D. helianthi* differed in their RE at 3-d after inoculation (Table 3.5). However, there were no significant differences in RE ($p \leq 0.05$) between the two *Diaporthe* species at 10-d and 14-d after inoculation. Based on visual symptoms, *D. gulyae* caused brown lesions extending upwards from the inoculation site, wilting of stem and leaves at the node closest to the site of inoculation 3-d after inoculation, and causing plant death. In contrast, *D. helianthi* caused tan to brown elongated lesions 10-d after inoculation and plant death did not occur. No symptoms were observed in the control plants.

Table 3.4. Test statistics for the effects of two species (*D. helianthi* and *D. gulyae*) and their respective isolates on Phomopsis stem canker of sunflower

	ANOVA type statistic (ATS)			
Effect	df_N (numerator degrees of freedom)	df_D (denominator degrees of freedom)	F	*p*-value
species	1	440	10.7	0.0012
isol(species)	18	440	78.83	< 0.0001
time	2	∞	690.91	<0.0001
species*time	2	∞	7.15	0.0009
isol*time(species)	36	∞	6.79	<0.0001

The repeated measures analysis also identified a significant interaction among *Diaporthe* isolates (within the two species) and time at $p < 0.0001$ (Table 3.4). Among *D. gulyae* isolates, significant differences in their RE was more evident at 3-d after inoculation as compared to 10-d and 14-d after inoculations (Appendix). For example, the RE of DG3 was lower than DG1 at 3-d after inoculation, however, these *D. gulyae* isolates were not significantly different in their RE at 10-d and 14-d after inoculations (Appendix). In contrast, among *D. helianthi* isolates, significant differences in their RE was more evident at 3-d and 10-d after inoculation as compared to 14-d after inoculation. For example, the RE of DH10 was lower than that of DH7, DH8 and DH9 at 3-d and 10-d after inoculation, however, the RE of the four *D. helianthi* isolates were not significantly different in their RE at 14-d after inoculation (Appendix).

Table 3.5. Median, mean rank and relative treatment effects for Phomopsis stem canker severity rating caused by two *Diaporthe* species on sunflower at 3-d, 10-d, and 14-d after inoculation

Species	Median disease rating[a]			Mean rank			Estimated relative effect (RE)[b]		
	3-d	10-d	14-d	3-d	10-d	14-d	3-d	10-d	14-d
D. gulyae	2.5	3.0	4.0	257.9	378.5	495.0	0.36 (0.32,0.39)	0.53 (0.49,0.56)	0.69 (0.66,0.71)
D. helianthi	2.0	3.0	3.0	194.2	370.2	370.2	0.27 (0.24,0.30)	0.51 (0.47,0.56)	0.65 (0.62,0.67)

[a] Disease severity ratings was evaluated at 3-d, 10-d, 14-d after inoculation using a 0 to 5 scale modified from Thompson *et al*. (2011), where; 0 = no discoloration; 1 = low level discoloration at site of inoculation; 2 = slight discoloration or lesion 1–2 mm in length; 3 = necrotic lesions 2–5 mm in length, some colored stem streaking, leaf wilting and twisting; 4 = lesions 5–10 mm in length, significant necrosis and dark colored stem streaking, leaf and plant wilting, twisting, stunting, and some lodging; 5 = lesions exceeding 10 mm in length, severe leaf necrosis, lodging or plant death.
[b] Relative treatment effects were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004).

Overall, given *D. helianthi* produced symptoms consistent with Phomopsis stem canker 10-d after inoculation as compared to *D. gulyae*, disease evaluations were performed at 10-d when

sunflower genotypes were screened for resistance to the two *Diaporthe* spp. in the subsequent experiment.

To complete Koch's postulates, *D. gulyae* was re-isolated from the inoculated sunflower plants and identity of the pathogen was confirmed via sequencing of the internal transcribed spacer (ITS) regions using primers ITS5 and ITS4 (White *et al*., 1990). The pathogen was not isolated from the control plants (data not presented).

Screening sunflower genotypes for resistance to the two *Diaporthe* species

Test statistics indicated no significant effect of experiment or interaction effect between experiment and *Diaporthe* species or interactions between experiment, *Diaporthe* species and sunflower genotypes in the overall development of Phomopsis stem canker on sunflowers (data not presented).

A significant interaction was observed between *Diaporthe* species and sunflower genotypes at $p < 0.0001$ indicating differences in the response of the sunflower genotypes to *D. gulyae* and *D. helianthi* (Table 3.6).

Table 3.6. Test statistics for the effects of the two species (*D. helianthi* and *D. gulyae*) on one USDA inbred, nine USDA PI and one commercial hybrid screened for Phomopsis stem canker resistance

	ANOVA type statistic (ATS)			
Effect	df_N (numerator degrees of freedom)	df_D (denominator degrees of freedom)	F	*p*-value
species	1	180	419.40	< 0.0001
genotypes	9	180	14.52	< 0.0001
species* genotypes	9	180	6.04	< 0.0001

Disease caused by the two *Diaporthe* species on the sunflower genotypes was evaluated at 10-d after inoculation using RE and their 95% CI (Table 3.7).

Table 3.7. Median, mean rank and relative treatment effects for Phomopsis stem canker severity rating on sunflower caused by *D. helianthi* and *D. gulyae* on one USDA inbred, nine USDA PI and one commercial hybrid at 10-d after inoculation

Species	Genotypes	Country of origin	Median disease rating[a]	Mean rank	Estimated relative effect (RE)[b]	Confidence interval (95%) for relative treatment effect	
						Lower limit	Upper limit
D. helianthi	HA 288		5.0	173	0.86	0.79	0.91
	PI 162784	Argentina	3.5	132	0.66	0.51	0.77
	PI 507894	Hungary	4.0	111	0.55	0.43	0.67
	PI 531389	Czech	3.5	122	0.61	0.50	0.71
	PI 219649	Austria	4.0	139	0.69	0.64	0.74
	PI 507911	Hungary	4.0	135	0.67	0.64	0.71
	PI 650523	Canada	5.0	168	0.84	0.72	0.91
	PI 650348	China	3.0	107	0.53	0.38	0.68
	PI 531366	Poland	4.0	144	0.72	0.65	0.77
	Novi Sad	Serbia	5.0	177	0.88	0.83	0.92
D. gulyae	HA 288		3.0	73	0.36	0.24	0.50
	PI 162784	Argentina	2.0	39	0.19	0.17	0.21
	PI 507894	Hungary	2.0	44	0.22	0.17	0.27
	PI 531389	Czech	2.0	54	0.27	0.20	0.35
	PI 219649	Austria	2.0	39	0.19	0.17	0.21
	PI 507911	Hungary	2.0	44	0.22	0.17	0.27
	PI 650523	Canada	4.0	139	0.69	0.64	0.74
	PI 650348	China	2.0	54	0.27	0.20	0.35
	PI 531366	Poland	3.0	79	0.39	0.29	0.51
	Novi Sad	Serbia	2.0	39	0.19	0.17	0.21

[a] Disease severity rating evaluated at 10-d after inoculation using a 0 to 5 scale modified from Thompson *et al*. (2011), where; 0 = no discoloration; 1 = low level discoloration at site of inoculation; 2 = slight discoloration or lesion 1–2 mm in length; 3 = necrotic lesions 2–5 mm in length, some colored stem streaking, leaf wilting and twisting; 4 = lesions 5–10 mm in length, significant necrosis and dark colored stem streaking, leaf and plant wilting, twisting, stunting, and some lodging; 5 = lesions exceeding 10 mm in length, severe leaf necrosis, lodging or plant death.
[b] Relative treatment effects were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004).

Among the nine genotypes, seven PIs specifically, 'PI 162784', 'PI 507894', 'PI 531389', 'PI 219649', 'PI 507911', 'PI 650348' and 'PI 531366' were significantly less susceptible to *D. helianthi* based on the RE produced by the pathogen as compared to that on cv. 'HA 288' (Table 3.7). In contrast, 'PI 162784', 'PI 219649', and 'Novi Sad Serbia' were significantly less susceptible to *D. gulyae* based on the RE produced by the pathogen as compared to that on cv. 'HA 288' (Table 3.7).

Discussion

In this study, four inoculation methods were evaluated on the basis of their capacity to cause Phomopsis stem canker on sunflowers using mycelial plug as the inoculum under greenhouse conditions. Based on the recovery of *D. helianthi* and its correlation with disease produced by the isolates at 14-d after inoculation, stem-wound method was adopted for subsequent experiments. Using this method, the aggressiveness of the *Diaporthe* species and isolates within species were compared under greenhouse conditions. Results suggest that while *D. gulyae* produced symptoms consistent with Phomopsis stem canker more quickly than *D. helianthi*, both are competent pathogens. The stem-wound method was also used to screen PI for resistance to the two *Diaporthe* species under greenhouse conditions and two PI lines, specifically 'PI 162784', and 'PI 219649' were significantly less susceptible to the two pathogens as compared to 'HA 288'.

The findings of the present study suggest that out of the four inoculation methods, stem-wound and wound-inoculation method may be the most effective. The rapid and uniform infection of host tissues in all inoculations achieved by these assays can be critical for aggressiveness studies of the *Diaporthe* species and their isolates, given that isolates of *D. helianthi* and *D. gulyae* vary in their aggressiveness characteristics such as lesion size (Thompson *et al*., 2011, Herr *et al*., 1983). In contrast, the petiole-based methods was prone to more injury during wounding and disease

produced by the *D. helianthi* isolates correlated low with the pathogen recovery compared to the stem-based methods. However, a major limitation of using stem-based inoculation method is that it will not enable evaluation of the leaf and petiole resistance, given leaf/petiole and stem resistance are inherited independently (Vear *et al*., 1997, Tourvieille *et al*., 1988).

This study was the first to compare the relative overall aggressiveness of the two *Diaporthe* spp. and results suggest *D. gulyae* was capable of causing significant necrosis, lodging and plant death as or more quickly than *D. helianthi*. Similarly, isolates of *D. gulyae* and *D. helianthi* varied significantly in their aggressiveness and the results are consistent with the findings of Vukojević *et al*. (2001) for *D. helianthi* in Europe and Thompson *et al*. (2011) for *D. gulyae* in Australia. *D. helianthi* is known to produce toxin metabolites, including phomozin, which can result in premature leaf senescence, plant wilting and lodging (Masirevic and Gulya, 1992). Although there are no studies that have characterized toxins produced by *D. gulyae*, the involvement of a toxin in the disease could explain these differences in terms of the varying aggressiveness between the two species and their isolates at specific times, for instance, 3-d for *D. gulyae* and 10-d after inoculations for *D. helianthi*. However, given both the *Diaporthe* species are present in the U.S., knowledge of the relative aggressiveness of the two *Diaporthe* species has important implications for the success of future breeding efforts and resistance deployment.

Among the nine genotypes that were screened for resistance to Phomopsis stem canker, seven genotypes were found to be more resistant than the susceptible check when inoculated with *D. helianthi*. However, only three genotypes were more resistant than the susceptible check when inoculated with *D. gulyae*. Although further experiments will be required to determine the number of loci and kinds of gene action governing resistance based on the genetic background of these genotypes, it appears that resistance to one *Diaporthe* species is not necessarily indicating

resistance to the other. For example, when using the susceptible genotype as a comparison, 'Novi Sad Serbia' was susceptible to *D. helianthi* but possessed some resistance to *D. gulyae,* while four genotypes appeared to be resistant to *D. helianthi* but not *D. gulyae*. If this is consistent among other isolates of the same species, it could have important implications for sunflower production worldwide and for managing Phomopsis stem canker using genetic resistance. Additionally, given that extensive genetic differences can occur between *Diaporthe* spp. such as *D. helianthi* strains from different geographical origins or even strains collected in one field (Says-Lesage *et al*., 2002), evaluation of local host genotypes with potential resistance to the two pathogens and testing them under multiple environments is essential.

Although greenhouse-based screening is faster, cost efficient, and often a more reliable way to ensure infection, it has limitations. Degener *et al*. (1999) suggested that no correlation exists between resistance in the leaf and stem, which means both these resistance factors are inherited independently. Because natural infection occurs through the leaves, the genotypes that were determined to be resistant in the field but not in the greenhouse may have had leaf resistance, which would have been undetectable using stem-based greenhouse methods. This explains as to why sunflower genotypes known to show resistance to Phomopsis stem canker in the field (Talukder *et al*., 2014), did not all show resistance in our greenhouse studies. In addition, we used mycelium as inoculum in our greenhouse tests and the conclusions on species effects or species by genotype interactions could be quite different if ascospores were used as inoculum as under natural conditions (Viguié *et al*., 1999, Bertrand and Tourvieille, 1987). In this study, the *D. helianthi* and *D. gulyae* isolates, which were determined to be most aggressive, were used for screening genotypes for resistance. The *D. gulyae* isolate appeared to lose aggressiveness when it was used to screen different genotypes for resistance; cv. 'HA 288' was not as susceptible to *D.*

gulyae as it was in the aggressiveness study when the two species were compared. This is a known phenomenon in *Diaporthe* spp., that they have reduced viability after repeated subculture or long-term storage (Baumgartner *et al*., 2013), as was the case for some of our isolates including *D. gulyae*. Consequently, it is important to use care when selecting and maintaining isolates to screen germplasm.

Despite the limitations in using the stem-based inoculation method, an advantage of using this method is that it can be used for screening plants for *Diaporthe* stem resistance because stem lesion development determines yield losses (Degener *et al*., 1999). In addition, stem inoculations may also be ideal for comparing pathogen comparisons. Disparities exist between greenhouse and field environments that may account for the differences in results between greenhouse and field screenings. Field screening can be carried out only once a year at most locations, is season dependent for disease development, and can be affected by uncontrollable environmental conditions such as temperature, humidity, and the simultaneous presence of other pathogens. However, greenhouse tests can be completed faster with less operational cost than screening for resistance in the field, and the likelihood of successful infection is higher in a controlled environment.

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CHAPTER THREE. *FUSARIUM* SPP. ASSOCIATED WITH SUNFLOWER STEM DISEASE IN THE UNITED STATES

Abstract

Fusarium is commonly regarded a minor pathogen on sunflowers (*Helianthus annuus* L.) in most production regions of the world. Although the economic implications of *Fusarium* spp. on sunflowers are not known in the United States, *Fusarium* pathogens are currently a serious economic problem only in Russia. In 2010, a total of 1,637 stalks were sampled for stem diseases in the Northern Great Plains (Minnesota, North Dakota and South Dakota). A total of 110 *Fusarium* isolates were recovered and identified to species level, using morphology. Phylogenetic analyses of repetitive-sequence-based polymerase chain reaction (rep-PCR) fingerprints and the translation elongation factor 1-alpha (EF-1α) gene revealed that *Fusarium* isolates from sunflowers represented strongly supported clades of eight species (75-100% similarity for rep-PCR and >95% posterior probability for EF-1α gene analyses), namely, *F. graminearum*, *F. proliferatum*, *F. culmorum*, *F. avenaceum*, *F. oxysporum*, *F. acuminatum*, *F. sporotrichioides* and *F. equiseti*. Pathogenicity studies in the greenhouse comparing the aggressiveness of eight *Fusarium* spp. using stem-wound method showed *F. sporotrichioides* to be the most aggressive and *F. graminearum* to be the least aggressive based on vascular discoloration. Our study also compared the aggressiveness of four isolates of three *Fusarium* spp. and six isolates representing six VCGs of *V. dahliae* (1, 2A, 2B, 4A, 4B and 6) using the root-dip method. There were no significant differences in estimated relative effects ($p \leq 0.05$) among the four isolates of three *Fusarium* spp. and *V. dahliae* isolates VCG1, VCG6 and VCG2A, however, VCG2B and VCG4B caused significantly more disease that all *Fusarium* species. The identification of eight *Fusarium*

species on sunflowers in the Northern Great Plains may be epidemiologically important for both sunflowers and crops rotated with sunflowers.

Introduction

Species of *Fusarium* are predominantly regarded as soil-borne fungi because of their abundance in soil and their frequent association with plant roots, as either pathogens or saprophytes (Leslie and Summerell, 2006). They are widespread and this may be attributed to the ability of these fungi to grow on a wide range of substrates and their efficient mechanisms for dispersal (Burgess, 1981). *Fusarium* is typically regarded as a minor pathogen in most sunflower (*Helianthus annuus* L.) production regions of the world (Gulya *et al*., 1997), but in some cases, it has been shown to cause yield loss. Yield losses up to 45% due to *Fusarium verticillioides* (Saccardo) Nirenberg (formerly called *F. moniliforme* Sheldon) have been reported in India (Aćimović, 1998) and unidentified *Fusarium* spp. are responsible for up to 80% loss in Russia (Gontcharov *et al*., 2006).

Many *Fusarium* species have been determined to cause diseases on sunflower, including; *Fusarium sporotrichioides* Bilai, *Fusarium acuminatum* Ellis and Everhart, *Fusarium solani* (Martius) Appel & Wollenweber emend. Snyder & Hansen, *Fusarium oxysporum* Schlecht. emend. Snyd & Hans., *F. verticillioides* (Saccardo) Nirenberg, *Fusarium poae* (Peck) Wollenw., *Fusarium equiseti* (Corda) Saccardo, *Fusarium culmorum* (W.G. Smith) Saccardo, and *Fusarium semitectum* Berkeley & Ravenel (Mathew *et al*., 2010, Antonova *et al*., 2002, Nahar and Mushtaq, 2006, Nahar and Mushtaq, 2007). Fusarium wilt of sunflower was first reported in the United States in Texas (Orellana, 1971). Despite the co-infection of *Macrophomina phaseolina* (Tassi) Goid., Orellana (1971) found that the unidentified *Fusarium* sp. was the primary etiological agent causing wilt. While yield loss to *Fusarium* spp. is not commonly documented, external plant

symptoms of *Fusarium* spp. infection are often general and relatively non-specific, and can include; wilting and seedling rot, stunting, yellowing, tip burning and reduction in growth on sunflowers (Mathew *et al*., 2010, Nahar and Mushtaq, 2006, Nahar and Mushtaq, 2007). These symptoms can be easily confused with other yield-limiting factors, including drought stress and other diseases such as Charcoal rot caused by *M. phaseolina* and Verticillium wilt caused by *Verticillium dahliae* Kleb. (Berglund, 2007).

Identification to species level using classical taxonomy is not always straightforward for *Fusarium* spp. This is in part because the cultural and morphological appearance of *Fusarium* species can be highly variable depending on the culture conditions (Yoder and Christianson, 1997), and a relatively high level of training is needed for accurate identification to species level. Molecular biology techniques such as sequencing the translation elongation factor 1-α (EF1-α) gene (Geiser *et al*., 2004), restriction fragment length polymorphism (RFLP) (Edel *et al*., 1995), repetitive-sequence-based polymerase chain reaction (Rep-PCR) (Edel *et al*., 1995), and restriction fragment analysis of PCR-amplified ribosomal intergenic spacers (IGS) have been used for the differentiation of *Fusarium* strains at the interspecific and intraspecific level (Edel *et al*., 1995). Rep-PCR is based on PCR-mediated amplification of DNA sequences located between specific interspersed repeated sequences termed BOX, REP, and ERIC elements in fungal genomes and have been used to characterize variability at inter-specific levels of several fungal genera, including *Diaporthe* spp. (Ash *et al*., 2010). However, Rep-PCR has not been used for identification and taxonomic purposes in *Fusarium* spp. at inter-specific level.

Several inoculation methods have been used to evaluate pathogenicity of *Fusarium* spp. of sunflowers such as soil inoculation methods and insertion of infested agar plugs into the stalks (Mathew *et al*., 2010, Nahar and Mushtaq, 2007, Nahar and Mushtaq, 2006, Antonova *et al*., 2002,

Ganacharya *et al*., 1978). Nahar and Mushtaq (2007) used a soil inoculation method wherein sunflower seeds were planted into sterilized soil mixed with corn meal-sand medium inocula of *Fusarium* spp. and studied symptoms produced by different *Fusarium* species. For example, sunflower plants inoculated with *F. equiseti, F. acuminatum, F. solani* and *F. subglutinans* showed the highest degree of wilting, while the highest levels of seedling rot was observed by *F. sporotrichioides* and *F. solani* (Nahar and Mushtaq, 2007). Antonova *et al*. (2002) compared sowing the seeds directly in artificially infested soil and injection of spore suspension into hypocotyls. Both the inoculation methods by Antonova *et al*. (2002) failed to distinguish varying degrees of resistance or susceptibility among sunflower genotypes. Root-dip inoculations have been used to evaluate Fusarium wilt, caused by *F. oxysporum*, in crops besides sunflowers such as in watermelon (*Citrullus lanatus* L., Egel and Martyn, 2007) and dry edible peas (*Pisum sativum* L., Egel and Martyn, 2007, Kraft and Haglund, 1978). Although there are no studies evaluating the efficacy of root-dip inoculation methods to assess Fusarium wilt and other *Fusarium* spp. on sunflowers, the method has been used to evaluate *V. dahliae* on sunflowers (Alkher *et al*., 2009).

In 2009, *Fusarium* infected sunflowers were first reported from Minnesota (Mathew *et al*., 2010). In 2010, a stem disease survey was conducted throughout the North Central Great Plains states of Minnesota (MN), North Dakota (ND) and South Dakota (SD). The survey was conducted at approximately R5 (mid-bloom). In 2010, 55 fields were arbitrarily selected, with no fields being closer than 5 km from a previously selected field (Mathew *et al*., unpublished [chapter 2], Table 2.1). In each field, one to two transects (rows) were randomly selected. In each transect, sunflower stalks exhibiting stem disease (such as Verticillium wilt and Phomopsis stem canker) or stem symptoms (wilting, lodging, and lesions of varying size and color) were arbitrarily selected until the end of the transect was reached. Over 1,600 stalk samples were collected from 55 fields,

returned to the laboratory, dissected and examined. Over 10% of those stalks had pink to purple discoloration in the pith, consistent with *Fusarium* infection. The objectives of this study were to 1) identify the species of *Fusarium* infecting sunflower stems, ii) determine if those species are pathogenic on sunflower, and iii) and compare the aggressiveness of those *Fusarium* species on sunflowers.

Materials and method

Fusarium isolation and identification by morphology

One hundred and two sunflower stalks with discoloration inside the stem consistent with *Fusarium* infection were received as part of a previous survey (unpublished data) (Table 4.1). Approximately 1 cm long pieces of symptomatic stalks (pink to dark brown discolored lesions) were cut from infected tissue and surface-sterilized before plating on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) amended with 0.02% streptomycin sulfate. Stem pieces were incubated at 25°C for 7-10 d under 16-h of alternating light and dark conditions. Cultures were scored for presence or absence of different fungi (including *Fusarium*). *Fusarium* isolates were hyphal-tipped, subcultured and identified, based on morphology (Leslie and Summerell, 2006). Hyphal tip transfer was done onto PDA plates and incubated at room temperature in a 16-h light/dark cycle until the formation of conidia. Conidial suspensions were prepared and streaked on plates with sterile water agar. As single germinating conidium was transferred to fresh PDA after 18-24 h and incubated at room temperature, these cultures were maintained as single spore isolates. *Fusarium* species were identified based on morphology, including the shapes and sizes of macroconidia and microconidia, the presence and absence of chlamydospores, by visual colony appearances and pigmentations on PDA (Leslie and Summerell, 2006).

Molecular identification and phylogenetics

In order to evaluate Rep-PCR as a tool for identification of *Fusarium* species recovered from sunflowers, 48 isolates were selected based on their geographic location throughout the Northern Great Plains to be representative of the species found in this study (Table 4.1). DNA was extracted from mycelial plugs of the 48 single-spored *Fusarium* isolates using a Cetyltrimethyl Ammonium Bromide (CTAB) protocol (Doyle and Doyle, 1987) and subjected to rep-PCR using primer sets corresponding to the BOX, ERIC, and REP elements. DNA from reference isolates that were previously confirmed for different *Fusarium* species by Mathew *et al*. (2008) and Bilgi *et al*. (2008), were also subjected to Rep-PCR genomic fingerprinting in the same PCR reaction as the unknown *Fusarium* isolates from sunflowers. The primer sets included BOX element 1A primer 1R (5`-CTACGGCAAGGCGACGCTGACG-3`); ERIC primers 1R (5`-ATGTAAGCTCCT GGGGATTCAC-3`) and 2I (5`-AAGTAA GTGACTGGGGTGAGCG-3`); and REP primers 1R (5`-IIIICGICGICATCIGGC-3`) and 2I (5`-ICGICTTATCIGGCCTAC-3`) (Versalovic *et al*., 1994). Reactions for all PCR amplifications were performed in a 25-µl mixture containing 20-30 ng/reaction of template DNA, 10 µM of each primer, 10 mM of each dNTPs, 5 units/µl of Taq DNA Polymerase (Qiagen, Valencia, CA), and 10x Qiagen PCR Buffer containing 15 mM $MgCl_2$ (Qiagen, Valencia, CA). The PCR cycling protocols were as follows: an initial denaturation at 94°C for 5 min, 35 cycles consisting of 94°C for 3 s, 92°C for 30 s, then either 40°C (REP primers) or 50°C (ERIC/BOX primers) for 1 min; extension at 72°C for 1 min; and a single final extension at 72°C for 10 min, followed by cooling at 4°C. To determine reproducibility of the results, all samples were subjected twice to independent PCR and gel electrophoresis. A 5-µl aliquot of each PCR product was run electrophoretically on a 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA) at a final concentration of 0.25×

to confirm amplification. The positions of fragments (bands) on each gel were normalized by using the 1-kb ladder from 298 to 5,090 bp as an external reference standard. All clearly visible bands were scored for analysis. Fragments amplified by each of the primers were scored as present (1) or absent (0). Data from each reaction was pooled for each *Fusarium* isolate. Distance matrix and cluster analysis was performed with Numerical Taxonomy and Multivariate Analysis System (NTSYS) v2.02j (Applied Biostatistics, Exeter Software, Setauket, New York). A similarity matrix was established using NTSYS with the similarity for qualitative data (SIMQUAL) function and the clustering was done using the unweighted pair group arithmetic mean method (UPGMA) (Sneath and Sokal, 1973) and a dendrogram was generated by using the SAHN (sequential, agglomerative hierarchical and nested clustering) module of NTSYS. The resulting dendrogram was compared to the similarity matrix using cophenetic correlation (COPH and MXCOMP) programs in NTSYS. Phylogenetic hypotheses were inferred from the matrix of characters scored from Rep-PCR amplifications using PAUP* v4.0b10 (Sinauer Associates, Inc., Sunderland, MA; Swofford, 2002), using 1,000 random repartitions (Felsenstein, 1985), with MAXTREES set to 5,000.

To confirm the molecular identification of the *Fusarium* species, 31 representative isolates were selected from Rep-PCR clusters (Table 4.2) and sequenced for inclusion in the phylogenetic analysis of the translation elongation factor 1-alpha (EF-1α) gene. The EF-1α gene was amplified using the primers EF1F/EF1R (O'Donnell *et al*., 1998). Reactions for PCR amplifications were performed in a 25-µl mixture containing 20-30 ng/reaction of template DNA, 10 µM of each primer, 10 mM of each dNTPs, 5 units/µl of Taq DNA Polymerase (Qiagen, Valencia, CA), and 10x Qiagen PCR Buffer containing 15 mM $MgCl_2$ (Qiagen, Valencia, CA). Cycle parameters included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C

for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (O'Donnell *et al.*, 1998). A 5-µl aliquot of each PCR product was run electrophoretically on a 1% agarose gel in agarose gels stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA, USA) at a final concentration of 0.25× to confirm amplification. All DNA samples were sequenced (McLab, San Francisco, CA and GenScript USA Inc., Piscataway, NJ) using the primers EF1F/EF1R (O'Donnell *et al.*, 1998). Forward and reverse sequences were edited and contigs were aligned using Bioedit (Hall, 1999). Analysis of the edited sequences was performed using Basic Local Alignment Search Tool nucleotide (BLASTN) searches at the GenBank nucleotide database (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) and Fusarium-ID database (Geiser *et al.*, 2004).

Table 4.1. Isolates of *Fusarium* species designated by isolate name and identity, state of origin, and host

Isolates[a]	State of origin	Host	Species identity[a]
Facum10MN-02	MN	*H. annuus*	*F. acuminatum*
Facum10ND-01	ND	*H. annuus*	*F. acuminatum*
Facum10ND-02	ND	*H. annuus*	*F. acuminatum*
Facum10ND-03	ND	*H. annuus*	*F. acuminatum*
Facum10ND-04	ND	*H. annuus*	*F. acuminatum*
Facum10ND-05	ND	*H. annuus*	*F. acuminatum*
Facum10SD-01	SD	*H. annuus*	*F. acuminatum*
Facum10SD-02	SD	*H. annuus*	*F. acuminatum*
Facum10SD-03	SD	*H. annuus*	*F. acuminatum*
Facum10SD-04	SD	*H. annuus*	*F. acuminatum*
Facum10SD-05	SD	*H. annuus*	*F. acuminatum*
Fculm10ND-01	ND	*H. annuus*	*F. culmorum*
Fequi10MN-01	MN	*H. annuus*	*F. equiseti*
Fequi10MN-02	MN	*H. annuus*	*F. equiseti*
Fequi10ND-01	ND	*H. annuus*	*F. equiseti*

[a] *Fusarium* isolates were hyphal-tipped and identified based on morphology (Leslie and Summerell, 2006).

Table 4.1. Isolates of *Fusarium* species designated by isolate name and identity, state of origin, and host (contd.)

Isolates[a]	State of origin	Host	Species identity[a]
Foxy10MN-01	MN	*H. annuus*	*F. oxysporum*
Fprol10MN-01	MN	*H. annuus*	*F. proliferatum*
Fsporo10MN-01	MN	*H. annuus*	*F. sporotrichioides*
Fsporo10MN-02	MN	*H. annuus*	*F. sporotrichioides*
Fsporo10MN-10	MN	*H. annuus*	*F. sporotrichioides*
Fsporo10ND-01	ND	*H. annuus*	*F. sporotrichioides*
Fsporo10ND-02	ND	*H. annuus*	*F. sporotrichioides*
Fsporo10ND-04	ND	*H. annuus*	*F. sporotrichioides*
Fsporo10ND-05	ND	*H. annuus*	*F. sporotrichioides*
Fsporo10SD-01	SD	*H. annuus*	*F. sporotrichioides*
Fsporo10SD-03	SD	*H. annuus*	*F. sporotrichioides*
Fsporo10SD-04	SD	*H. annuus*	*F. sporotrichioides*
Gaven10ND-01	ND	*H. annuus*	*F. avenaceum*
Gaven10ND-02	ND	*H. annuus*	*F. avenaceum*
Gaven10SD-01	SD	*H. annuus*	*F. avenaceum*
Gaven10SD-02	SD	*H. annuus*	*F. avenaceum*
Gzeae10MN-01	MN	*H. annuus*	*F. graminearum*
Gzeae10MN-02	MN	*H. annuus*	*F. graminearum*
Gzeae10ND-01	ND	*H. annuus*	*F. graminearum*
Gzeae10ND-02	ND	*H. annuus*	*F. graminearum*
Gzeae10ND-03	ND	*H. annuus*	*F. graminearum*
Gzeae10SD-01	SD	*H. annuus*	*F. graminearum*
Gzeae10SD-02	SD	*H. annuus*	*F. graminearum*
Gzeae10SD-03	SD	*H. annuus*	*F. graminearum*
F. avenaceum	ND	*Pisum sativum* L.	Mathew *et al*. (2008)
F. oxysporum	ND	*Pisum sativum* L.	Mathew *et al*. (2008)
F. acuminatum	ND	*Pisum sativum* L.	Mathew *et al*. (2008)
F. equiseti	ND	*Pisum sativum* L.	Mathew *et al*. (2008)
F. sporotrichioides	ND	*Pisum sativum* L.	Mathew *et al*. (2008)
F. solani	ND	*Pisum sativum* L.	Mathew *et al*. (2008)
F. graminearum	ND	*Phaseolus vulgaris* L.	Bilgi *et al*. (2008)
F. culmorum	ND	*Pisum sativum* L.	Mathew *et al*. (2008)
F. redolens	ND	*Pisum sativum* L.	Mathew *et al*. (2008)

[a] *Fusarium* isolates were hyphal-tipped and identified based on morphology (Leslie and Summerell, 2006).

Table 4.2. List of *Fusarium* isolates from sunflowers characterized using EF1-α gene

Isolates[a]	State of origin	Species identity
Gzeae10MN_01	MN	*F. graminearum*
Gzeae10SD_01	SD	*F. graminearum*
Gzeae10ND_01	ND	*F. graminearum*
Fculm10ND_01	ND	*F. culmorum*
Fculm10SD_01	SD	*F. culmorum*
Fculm10ND_02	ND	*F. culmorum*
Fsporo10MN_01	MN	*F. sporotrichioides*
Fsporo10ND_04	ND	*F. sporotrichioides*
Fsporo10MN_02	MN	*F. sporotrichioides*
Fsporo10ND_01	ND	*F. sporotrichioides*
Fsporo10SD_03	SD	*F. sporotrichioides*
Fsporo10SD_01	SD	*F. sporotrichioides*
Fsporo10SD_04	SD	*F. sporotrichioides*
Fequi10MN_01	MN	*F. equiseti*
Fequi10ND_01	ND	*F. equiseti*
Fequi10MN_02	MN	*F. equiseti*
Gaven10SD_01	SD	*F. avenaceum*
Gaven10ND_01	ND	*F. avenaceum*
Gaven10SD_02	SD	*F. avenaceum*
Facum10MN_02	MN	*F. acuminatum*
Facum10SD_01	SD	*F. acuminatum*
Facum10SD_03	SD	*F. acuminatum*
Facum10ND_05	ND	*F. acuminatum*
Facum10ND_03	ND	*F. acuminatum*
Foxy10MN_01	MN	*F. oxysporum*
Foxy10MN_02	MN	*F. oxysporum*
Foxy10SD_01	SD	*F. oxysporum*
Foxy10ND_01	ND	*F. oxysporum*
Fprol10MN_01	MN	*F. proliferatum*
Fprol10MN_02	MN	*F. proliferatum*
Fprol10MN_03	MN	*F. proliferatum*

[a] Isolates are a subset of 110 *Fusarium* isolates recovered from sunflowers, and were chosen as representatives for species-level identification.

Fungi were identified based on top BLAST results (lowest e-value, highest score, and greatest identity). Isolates that were found to have more than 95% identity with *Fusarium*

sequences in GenBank and have an e-value less than e-10 in the BLASTN searches were used for phylogenetic analysis.

To characterize *Fusarium* isolates to species by phylogenetic analyses, 57 references sequences (Alvarez *et al*., 2012, O'Donnell *et al*., 2012, Yli-Mattila *et al*., 2011, Balmas *et al*., 2010, O'Donnell *et al*., 2009, Nalim *et al*., 2009, Geiser *et al*., 2004, O'Donnell *et al*., 1998) were obtained from the NCBI and Fusarium-ID database. The sequences representing the outgroup *Fusarium solani* was obtained from GenBank (NCBI Accession number JF740849, JF740866 and JF740858) and these sequences were obtained from O'Donnell *et al*. (2012).

The EF-1α sequences of *Fusarium* isolates were aligned, using the default parameters of ClustalX (Thompson *et al*., 1997) and adjusted manually by visual examination, using the Molecular Evolutionary Genetics Analysis (MEGA) software v5 (Tamura *et al*., 2011) prior to being exported as NEXUS files for subsequent analyses. The EF-1α phylogeny was estimated with Bayesian inference (BI) with MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) under the estimated model of evolution. A maximum likelihood statistical method implemented in MEGA was first used to determine the best-fit nucleotide substitution models for 89 EF-1α sequences based on Bayesian Information Criterion (BIC); BIC values revealed that the best substitution model was Kimura 2-parameter (K2) with discrete gamma (+G) shape distribution (Kimura, 1980). For BI analyses, two simultaneous runs were conducted with Markov chain Monte Carlo (MCMC) chains each, default priors, a conservative burn-in of 25% and tree sampling every 100 generations. Four parallel MCMC chains were run for 1,000,000 generations; a burn-in of 1100 generations was found to be sufficient to achieve stationary model parameters using Tracer v1.4.1 (Rambaut and Drummond, 2007). Within each run, the average standard deviation of split frequencies (ASDSF) and the potential scale reduction factor (PSRF) statistics from MrBayes were used to

evaluate topological and branch-length convergence, respectively. The Bayesian probabilities (PP) for each node were estimated from the resulting 50% majority-rule consensus tree and nodes with 95% or greater PPs was considered significant (Wilcox *et al.*, 2002). Each of the Bayesian MCMC analyses was run at least twice to confirm the consistency of the results. Phylogenetic trees inferred using MrBayes was observed in FigTree v1.3.1 (Rambaut, 2009).

Determination of aggressiveness of *Fusarium* spp.

The aggressiveness of *Fusarium* species were compared, using the stem-wound method under greenhouse conditions to determine if these species can cause stem disease on sunflower (Mathew *et al*., unpublished [chapter 3]). Briefly, three seeds of the susceptible confection hybrid 'CHS RH3701' were sown into 7.5 liter plastic circular pots filled 75% full with a potting mix (Sunshine Mix # 1, Sun Grow Horticulture Products, Belleview, WA). The pots were placed on greenhouse benches at 22 to 25°C under a 16-h light/dark cycle and watered alternate days. When sunflower plants were at growth stage V4 to V6, stems were wounded with micropipette tips (100 μl) on the 2nd internode. Three isolates of each of the eight *Fusarium* spp. identified in this study were randomly selected as representative species. Inoculum was prepared by extracting a mycelial plug (4 mm diameter) with the ends of disposable micropipette tips from the margin of a 10-d PDA culture. The mycelial plug was placed over the wounded stem. Control plants were wounded with micropipette tips on the stem and petiole as for the treated plants and non-infested PDA plug was placed on the wound. All plugs were attached to the wounds with Parafilm to avoid a rapid dehydration.

The study was designed as a completely randomized design, with each treatment (isolates) replicated five times (five plants) and the experiment was repeated twice. Data from the two

experiments were combined after a test for homogeneity of variance (within and between experiments).

Disease severity was calculated as the percentage of host tissues covered by external lesion or damaged internally by the disease. Internode length and lesion length measured 14-d after inoculation was used to calculate the lesion length as a percentage of the internode length. The extent of vascular discoloration was measured length-wise 14-d after inoculation and calculated as a percentage of the internode length. Aggressiveness was analyzed using nested ANOVA (Schultz, 1955) in the SAS PROC GLM procedure to test for significant effect of species and isolates within species. Analysis of variance (ANOVA) was performed on SAS v9.3 (SAS Institute, Cary, NC). Mean comparisons were based on least significant difference (LSD) at $p \leq 0.05$.

Pearson correlation coefficient (Moore and McCabe, 1989) was used as a measure of the strength of association of disease severity values between lesion on the internode and vascular discoloration. Correlation analysis was performed using the PROC CORR procedure on SAS.

In addition to comparing aggressiveness of the different *Fusarium* species, all pathogens were re-isolated from host plants in order to complete Koch's postulates. Re-isolation was done according to techniques previously described. Identification of each *Fusarium* species was done morphologically and by EF-1α gene sequencing.

Comparison of aggressiveness of *Fusarium* spp. and *V. dahliae*

In an attempt to determine the competency of *Fusarium* species as a wilt pathogen, the aggressiveness of *Fusarium* spp. was compared to *Verticillium dahliae* (causal agent of Verticillium wilt on sunflowers). Both *Fusarium* spp. and *V. dahliae* are soil-borne fungi that cause vascular wilt disease and grow in the water-conducting tissues of the plant. For this

experiment, the root-dip method was adopted. The method has been used for *V. dahliae* on sunflowers (Alkher *et al*. 2009), but to the best of our knowledge, has not been used for *Fusarium* spp. Four *Fusarium* isolates (two *F. sporotrichioides*, one *F. equiseti* and one *F. oxysporum*) and six *V. dahliae* isolates representing six different vegetative compatibility groups (VCG), namely, VCG1B, VCG2A, VCG2B, VCG4A, VCG4B and VCG6 were compared for aggressiveness under greenhouse conditions.

Inoculum was prepared by growing cultures of *Fusarium* spp. and *V. dahliae* on PDA. *Verticillium dahliae* isolates were incubated at 25°C in the dark for 3-wk and *Fusarium* spp. isolates were incubated at 25°C under alternating 16-h light and dark conditions. Inoculum from both pathogens was prepared by flooding the plates with sterile distilled water and rubbing gently with a sterilized glass rod. The resulting suspension was filtered through double layers of cheesecloth, spores were counted with a hemocytometer and diluted to 10^6 conidia/ml. Seeds of the confection inbred 'HA 288' were sown in Cone-tainers (165 ml, Ray Leach "Cone-tainers"™, Stuewe & Sons, Inc. Tangent, Oregon) filled 90% full with potting mix. The pots were placed on the greenhouse benches at 22 to 25°C under a 16-h light and dark cycle and watered alternate days. Two week-old seedlings (V2-V3 growth stage) of susceptible were gently removed from the Cone-tainers and soil was washed off under running tap water. Following the rinsing, a few mm of the root tips were trimmed, and trimmed roots were dipped for approximately 10 min into the inoculum solutions prepared from each isolate (method modified from Alkher *et al*., 2009 and Radi and Gulya, 2007). Control plants were handled identically except that sterile distilled water was substituted for the conidial suspension. The seedlings were transplanted into 7.5-liter plastic pots with pasteurized soil (Sunshine Mix # 1) and returned to greenhouse conditions.

The experiment was designed as a completely randomized design with six plants (V4-V6 stage) evaluated per treatment and the experiment was repeated twice. The experimental unit is the single plant in each pot. Plants were assessed for vascular discoloration at 49-d after inoculation on a scale of 0 to 5 according to Alkher *et al*., (2009). Analysis of data based on observed ranks was performed in SAS v9.3 (SAS Institute, Cary, NC). The ordinal data from the disease rating scale did not have a normal distribution; therefore they were analyzed using the nonparametric procedure of Brunner *et al*. (2002) as described by Shah and Madden (2004). Preliminary data analysis showed that the results of both experimental runs were very similar, and so the data from both runs were combined for the final analysis. The overall effect of treatments on disease severity was determined by the analysis of variance type statistic (ATS) of ranked data (Singer *et al*., 2004). The ATS has an approximate F distribution (with df_N [numerator] and df_D [denominator] degrees of freedom) under the null hypothesis. The PROC RANK procedure in SAS was used to obtain midranks followed by PROC MIXED to generate relative effects (REs). The null hypothesis (H_o) is that the RE of all treatments are the same, while the alternative hypothesis (H_A) is that at least one of the relative treatment effects (RE) is different from the rest from the other treatments. RE, defined by means of probabilities derived from the marginal distribution functions of disease severity, are generated by the equation: $RE = (R – 0.5)/N$; where R is the mean treatment ranking and N is the total number of observations in the data analysis. Confidence intervals were calculated using LD_CI macro in SAS at $p \leq 0.05$ (Brunner *et al*., 2002).

Results

Fusarium isolation and identification by morphology

A total of 110 *Fusarium* isolates were recovered from infected sunflower stalks exhibiting internal symptoms consistent with *Fusarium* (Table 4.3). Eight *Fusarium* species were identified,

of which *Fusarium sporotrichioides* Sherb. (37.3%), *Fusarium acuminatum* Ellis & Everh. (33.6%), and *Fusarium graminearum* Schwabe (10.0%) were the most common, while *Fusarium avenaceum* (Fr.) Sacc., *Fusarium oxysporum*, *Fusarium proliferatum* (Matsush.) Nirenberg, *Fusarium equiseti* (Corda) Saccardo, and *Fusarium culmorum* (W.G. Sm.) McAlpine were each found 2.7 to 4.5% of the isolations (Table 4.3).

Table 4.3. Number and origin of *Fusarium* species identified from sunflowers using morphology (Leslie and Summerell, 2006)

	Number of *Fusarium* isolates recovered by state				
Fusarium species	MN (Number of stalks =21)	ND (Number of stalks = 33)	SD (Number of stalks = 48)	Total	*Fusarium* isolation (%)
F. acuminatum	2	11	24	37	33.6
F. equiseti	2	1	1	4	3.6
F. avenaceum	2	0	3	5	4.5
F. culmorum	0	2	3	5	4.5
F. graminearum	3	5	3	11	10.0
F. proliferatum	3	0	0	3	2.7
F. oxysporum	2	2	0	4	3.6
F. sporotrichioides	10	14	17	41	37.3
Total	24	35	51	110	

Molecular identification and phylogenetics

The size of the amplification products from Rep-PCR, ranged from approximately 100 bp up to 4,000 bp. Each primer set generated approximately 25 to 30 bands visible on the agarose gel. When fingerprint patterns of the 48 samples generated in the two independent runs were compared, using Cophenetic correlation coefficient (*r* value), and an *r* value of 0.85 to 0.90 was found, which is consistent with the correlation results from other Rep-PCR studies (Vinuesa *et al*., 1998). The correlation analyses for the UPGMA dendogram of the combined BOX, ERIC, and REP patterns returned an *r* value of 0.946 (>0.6), indicating a very good fit of the dendrogram to the data (Versalovic *et al*., 1991).

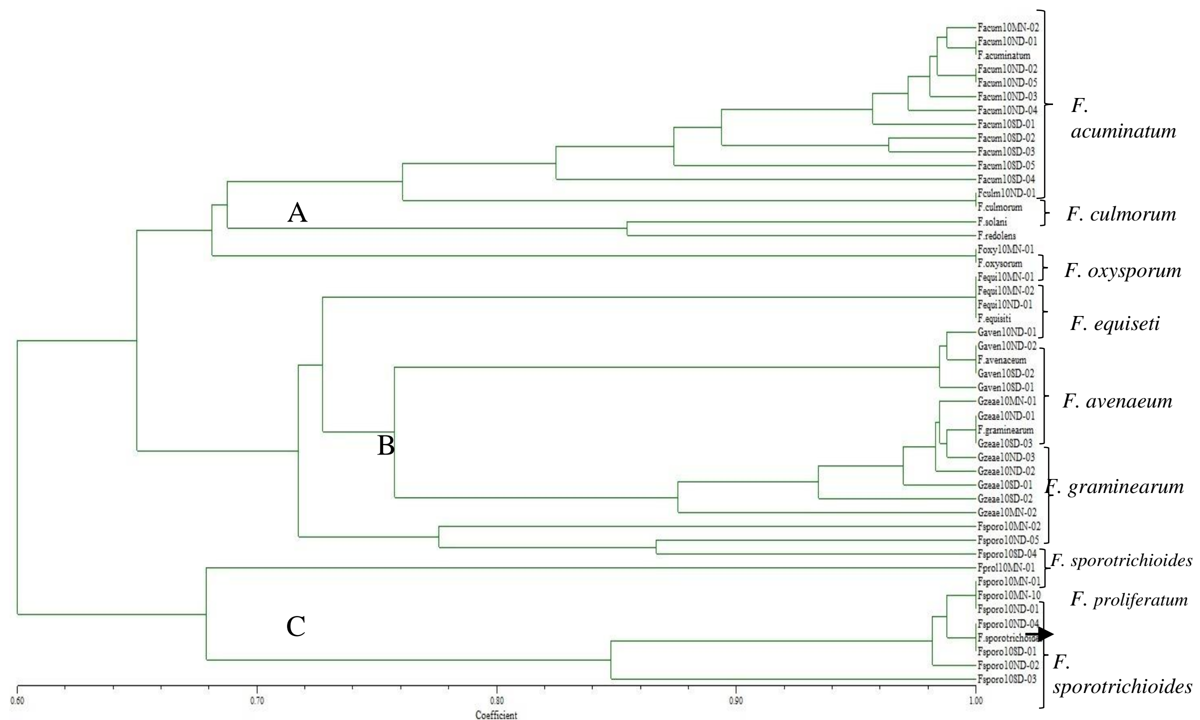

Fig. 4.1. Similarity of isolates of *Fusarium* spp. based on the presence and absence of bands generated using primers corresponding to repetitive extragenic palindromic sequences (REP), BOX1A sequences (BOX), and enterobacterial repetitive intergenic consensus sequences (ERIC). Three major groupings are designated A to C.

Analysis of the combined BOX, ERIC, and REP patterns revealed that the *Fusarium* isolates and reference isolates clustered together into three major groups designated A to C (Fig. 4.1). Group A contained two separate clusters of *F. oxysporum* and another cluster containing isolates of *F. acuminatum*, *F. culmorum*, *F. solani* and *F. redolens* Wollenw. that shared a similarity of approximately 68%. Isolates of *F. acuminatum* and *F. culmorum* were separated by approximately 76% and grouped with the reference isolates. There were no isolates from our collection that were similar to the reference isolates of *F. solani* and *F. redolens*. Group B contained two separate clusters of *F. sporotrichioides* and another cluster containing subclusters of *F. equiseti*, *F. graminearum* and *F. avenaceum* isolates. While *F. equiseti* isolates formed a tight cluster of approximately 100% with the reference isolate, *F. avenaceum* isolates (including

the reference) formed a cluster with approximately 95 to 100% similarity and isolates of *F. graminearum* (including the reference) formed a cluster with approximately 88-100% similarity. Group C contained two separate clusters of *F. sporotrichioides* and *F. proliferatum*. Isolates of *F. sporotrichioides* (including the reference) formed a cluster with approximately 85-100% similarity.

Approximately 700 bp of the EF1-α region was amplified from a total of 32 *Fusarium* isolates and used to query the GenBank database directly. However, only approximately 600 bp could be used to compare with the GenBank-retrieved sequences. A BLASTN search of Mycobank was performed for the EF1-α sequences of the *Fusarium* isolates. The suspected *F. graminearum* isolates matched *Gibberella zeae* strain NRRL29149 (Accession # DQ459738) with identities = 626/626(100%) and gaps = 0/626 (0%); *F. culmorum* isolates matched *Fusarium culmorum* strain NRRL 46656 (Accession # GU250558) with identities = 621/621(100%) and gaps = 0/621(0%); *F. sporotrichioides* isolates matched *Fusarium sporotrichioides* strain CBS 534.96 (Accession # EU128185) with identities = 552/552(100%) and gaps = 0/552(0%); *F. equiseti* isolates matched *Fusarium equiseti* strain NRRL 36136 (Accession # GQ505594) with identities = 630/630(100%) and gaps = 0/630(0%); *F. oxysporum* isolates matched *Fusarium oxysporum* strain NRRL 46589 (Accession # FJ985438) with identities = 614/614(100%) and gaps = 0/614(0%); *F. proliferatum* isolates matched *Fusarium proliferatum* strain NRRL 32155 (Accession # FJ538242) with identities = 626/626(100%) and gaps = 0/626(0%); *F. avenaceum* isolates matched *Gibberella avenacea* voucher FRC R-0048 (Accession # EU357809) with identities = 626/626(100%) and gaps = 0/626(0%) and *F. acuminatum* isolates matched *Fusarium acuminatum* isolate R-6678 (Accession # FJ154737) with identities = 628/628 (100%) and gaps = 0/628 (0%).

Since strains within a particular species had identical genotypes, only representative isolates from each of the *Fusarium* species were included in the phylogenetic analyses.

For characterizing sequences of the 31 *Fusarium* isolates from sunflowers, Bayesian and maximum likelihood analyses produced similar topologies in analyses of the EF1α gene and unknown isolates grouped with reference sequences into distinct clades (Fig. 4.2).

Two major clades (Group A and Group B) were identified in the resulting BI tree (Fig. 4.2). Within Group A, 24 of the 31 isolates were distributed into two main subclades (1 and 2, with 100% bootstrap support). Within subclade 1 of Group A, three *Fusarium* isolates clustered together with 10 reference sequences of *F. graminearum*, three isolates clustered together with five reference sequences of *F. culmorum*, seven isolates clustered together with six reference sequences of *F. sporotrichioides*, and three *Fusarium* isolates clustered together with five reference sequences of *F. equiseti*. Each of these clusters had a PP value of 100%. Within subclade 2 of Group A, three *Fusarium* isolates clustered together with eight reference sequences of *F. avenaceum*, and five isolates clustered together with five reference sequences of *F. acuminatum*. While the *F. avenaceum* cluster had a PP value of 99%, *F. acuminatum* cluster had a PP value of 100%. Within Group B, the remaining seven of the 31 *Fusarium* isolates from sunflowers were distributed into the subclades *F. oxysporum* and *F. proliferatum* (with 100% PP respectively). Four *Fusarium* isolates clustered together with nine reference sequences of *F. oxysporum*, and three isolates clustered together with six reference sequences of *F. proliferatum* in the BI tree (Fig. 4.2).

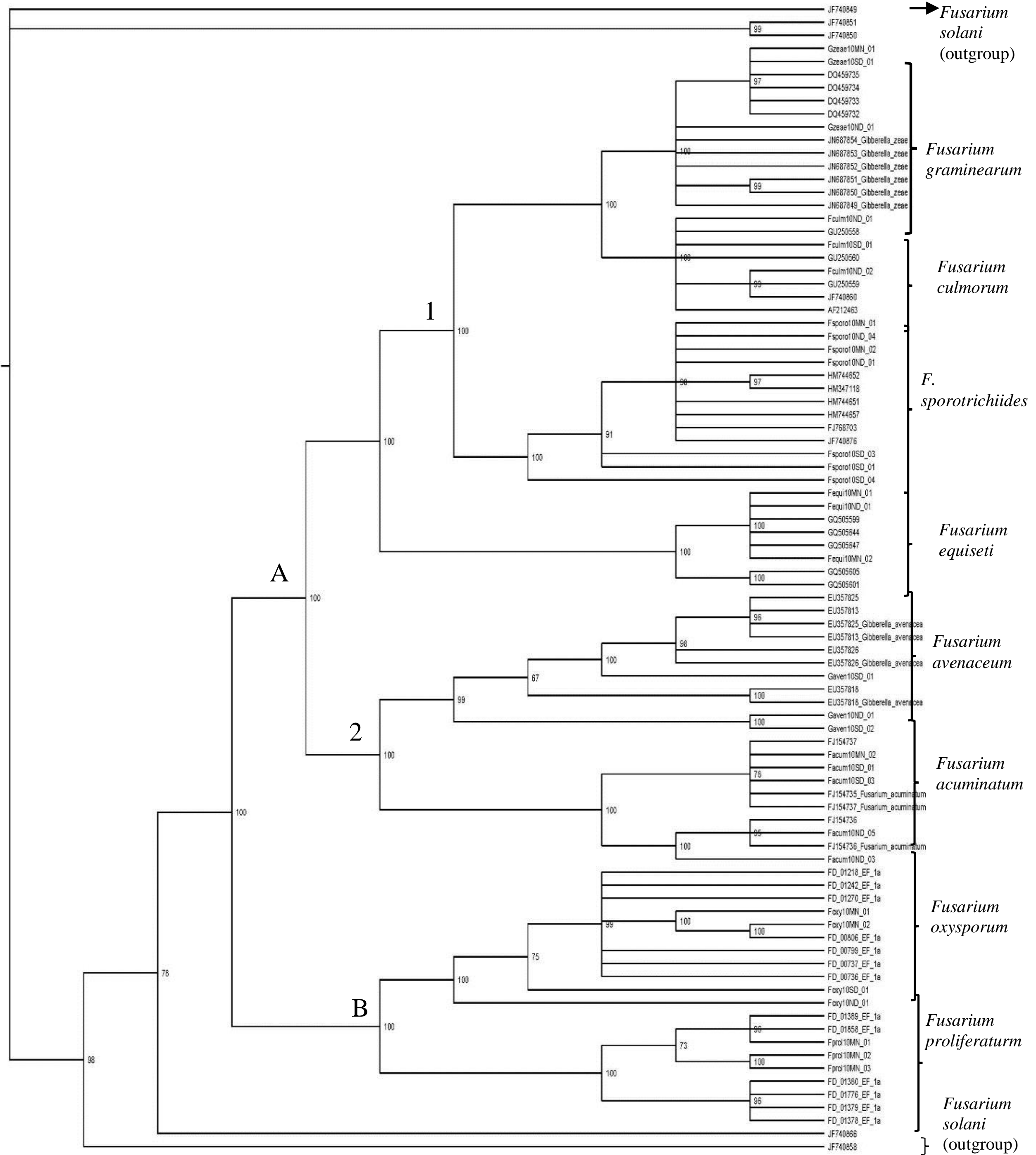

Fig. 4.2. Phylogenetic tree resulting from Bayesian inference analysis of the EF1-α region of the *Fusarium* isolates from sunflower. Bayesian posterior probabilities (>0.7) for the Bayesian analyses are indicated at the internodes. The tree is rooted with the outgroup *Fusarium solani* (NCBI Accession number JF740849, JF740866 and JF740858).

Determination of aggressiveness of *Fusarium* spp.

Test statistics indicated no significant effect of experiment or interaction effect between experiment and *Fusarium* species or interactions between experiment, *Fusarium* species and isolates within species in the overall development of Fusarium stem disease on sunflowers (data not presented).

Table 4.4. Nested ANOVA results for disease severity caused by isolates of the different *Fusarium* spp. on sunflower cv. 'CHS RH3701' using stem wound inoculation method in greenhouse evaluations

Sources of variation	df (degrees of freedom)	Sum of Squares	Mean Square	F-value	*p*-value
species	7	74971.19	10710.17	26.65	<0.0001
Isolate (species)	16	47505.48	2969.09	7.39	<0.0001
Error	216	86803.65	401.86		
Total	239	209280.33			

Table 4.5. Aggressiveness of eight *Fusarium* spp. on sunflower cv. 'CHS RH3701' under greenhouse conditions using stem-wound inoculation method

Fusarium species	Lesion length at 14-d[a]	Vascular discoloration at 14-d
	(% internode length)[b]	(% internode length)[c]
F. acuminatum	17.6 b	60.1 b
F. equiseti	63.2 a	83.3 a
F. avenaceum	34.0 b	55.1 bc
F. culmorum	19.4 bc	40.8 cd
F. graminearum	17.4 c	26.3 d
F. proliferatum	15.9 c	26.7 d
F. oxysporum	15.5 c	32.9 d
F. sporotrichioides	53.4 a	100.0 a
Non-infested control	10.2 c	5.7 d
LSD ($p \leq 0.05$)	15.8	18.8
$p > F$	< 0.0001	< 0.0001

[a] The results of two experiments with data combined for analysis based on the result taking three isolates of each species.
[b] expressed as a percentage of lesion length and internodal length.
[c] expressed as a percentage of vascular discoloration and internodal length.

All plants inoculated with *Fusarium* isolates resulted in disease ratings greater than the control (significant at $p \leq 0.05$) over the 2-week assessment period. *Fusarium* isolates caused brown-black lesions upwards from the inoculation site, wilting of stem and leaves at the node closest to the site of inoculation, with lesions expanding rapidly upwards causing plant death (at 14-d). When infected stems are split, brown to black streaks are evident in the vascular system. No symptoms were observed in the control plants.

To complete Koch's postulates, re-isolation of *F. graminearum*, *F. proliferatum*, *F. culmorum*, *F. avenaceum*, and *F. equiseti* was re-isolated from the inoculated sunflower plants, cultured and identities confirmed using morphology and EF1-α sequencing using the primers EF1F/EF1R (O'Donnell *et al*., 1998). No pathogens were isolated from the control plants.

Among species, *F. sporotrichioides* and *F. equiseti* produced significantly higher lesion length and vascular discoloration ($p \leq 0.05$) indicating they were more aggressive than the other species (Table 4.5). There were no significant differences in lesion length and vascular discoloration ($p \leq 0.05$) among *F. oxysporum*, *F. proliferatum* and *F. graminearum* and was comparable to the non-infested control (Table 4.5).

Lesion lengths produced by isolates of *F. acuminatum*, *F. proliferatum* and *F. graminearum* did not vary significantly within species ($p \leq 0.05$); however, vascular discoloration produced by isolates of *F. acuminatum* and *F. proliferatum* did vary within species ($p \leq 0.05$) (Table 4.6). Lesion length and vascular discoloration produced by isolates of *F. avenaceum*, *F. culmorum*, *F. oxysporum*, and *F. equiseti* varied significantly within species ($p \leq 0.05$) (Table 4.6). Lesion length produced by isolates of *F. sporotrichioides* varied significantly within species ($p \leq 0.05$), however, vascular discoloration did not vary within species ($p \leq 0.05$) (Table 4.6).

Table 4.6. Lesion length and vascular discoloration produced by isolates of eight *Fusarium* species on sunflower under greenhouse conditions using stem-wound method

Fusarium species	Isolate	Lesion length[a] (% internode)	Vascular discoloration[b] (% internode)
F. acuminatum	Facum10MN-02	15.60 a	56.10 b
	Facum10ND-01	13.25 a	39.88 b
	Facum10SD-01	24.02 a	84.47 a
	LSD ($p > F$)	12.25 ($p = 0.09$)	25.81 ($p = 0.0008$)
F. equiseti	Fequi10MN-01	100.00 a	68.33 b
	Fequi10MN-02	50.62 b	81.64 ab
	Fequi10ND-01	39.23 b	100.00 a
	LSD ($p > F$)	28.45 ($p < 0.0001$)	26.77 ($p = 0.02$)
F. oxysporum	Foxy10MN-01	29.55 a	51.26 a
	Foxy10ND-01	9.62 b	13.89 b
	Foxy10SD-01	7.60 b	33.75 ab
	LSD ($p > F$)	15.91 ($p = 0.003$)	30.02 ($p = 0.02$)
F. sporotrichioides	Fsporo10MN-01	55.81 ab	100.00 a
	Fsporo10ND-01	27.76 b	100.00 a
	Fsporo10SD-01	76.67 a	100.00 a
	LSD ($p > F$)	31.34 ($p = 0.003$)	0.00
F. avenaceum	Gaven10ND-01	45.47 a	81.14 a
	Gaven10SD-01	14.16 b	27.82 b
	Gaven10SD-02	42.37 ab	56.57 ab
	LSD ($p > F$)	30.93 ($p = 0.03$)	33.92 ($p = 0.002$)
F. graminearum	Gzeae10MN-01	15.12 a	23.37 a
	Gzeae10ND-01	24.68 a	17.30 a
	Gzeae10SD-01	12.49 a	38.49 a
	LSD ($p > F$)	20.97 ($p = 0.33$)	24.00 ($p = 0.10$)
F. culmorum	Fculm10ND-01	33.92 a	13.31 b
	Fculm10ND-02	10.67 b	57.47 a
	Fculm10SD-01	13.61 b	51.73 a
	LSD ($p > F$)	11.60 ($p < 0.0001$)	22.77 ($p < 0.0001$)
F. proliferatum	Fprol10MN-01	18.05 a	39.64 a
	Fprol10MN-02	8.00 a	5.37 b
	Fprol10MN-03	21.84 a	35.21 ab
	LSD ($p > F$)	15.06 ($p = 0.08$)	32.61 ($p = 0.03$)

[a] expressed as a percentage of lesion length and internodal length.
[b] expressed as a percentage of vascular discoloration and internodal length.

Pearson correlation coefficients indicate that a strong and significant correlation (n= 240; r= 0.61; $p < 0.0001$) exists between disease severity values expressed as a percentage lesion on the internode and disease severity values expressed as a percentage vascular discoloration.

Comparison of aggressiveness of *Fusarium* spp. and *V. dahliae*

Test statistics indicated no significant effect of experiment or interaction effects between experiment and treatments in the overall development of disease on sunflowers (data not presented). A significant effect of treatments was observed at $p < 0.0001$ (data not presented). Disease caused by the treatments was evaluated at 49-d after inoculations using RE and their 95% CI (Table 4.7).

Table 4.7. Disease rating of four isolates of *Fusarium* spp. and six isolates of *V. dahliae* representing different VCGs on sunflower under greenhouse conditions

Species	Isolate	Median disease rating[a]	Mean rank	Estimated relative effect (RE)[b]	Confidence interval (95%) for relative treatment effect	
					Lower limit	Upper limit
F. sporotrichioides	Fsporo10MN-01	1.0	50.5	0.49	0.32	0.66
F. sporotrichioides	Fsporo10ND-01	1.0	46.5	0.45	0.41	0.49
F. oxysporum	Foxy10MN-01	1.0	46.5	0.45	0.41	0.49
F. equiseti	Fequi10ND-01	1.0	51.4	0.50	0.41	0.59
V. dahliae (VCG4B)	VdUSA10-407	5.0	93.5	0.90	0.85	0.93
V. dahliae (VCG2B)	VdUSA10-72	5.0	93.5	0.91	0.86	0.94
V. dahliae (VCG4A)	VdUSA10-414	2.0	67.5	0.66	0.53	0.76
V. dahliae (VCG1)	VdUSA10-18	1.0	34.8	0.34	0.22	0.48
V. dahliae (VCG6)	VdUSA10-17	1.0	46.5	0.45	0.41	0.49
V. dahliae (VCG2A)	VdUSA10-16	1.0	51.4	0.50	0.41	0.59
Water control		0.0	11.5	0.11	0.09	0.13

[a] Plants were assessed for vascular discoloration at 49-d after inoculation using the root-dip method on a scale of 0 to 5 according to Alkher *et al*. (2009).
[b] Relative treatment effects were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004).

All plants inoculated with *Fusarium* and *Verticillium* isolates resulted in disease ratings greater than the water control (significant at $p \leq 0.05$) over the 49-d assessment period; the estimated relative effects ranged from 0.11 up to 0.91 (Table 4.7). Among the treatments, *V. dahliae* VCG4B isolate VdUSA10-407 and *V. dahliae* VCG2A isolate VdUSA10-16 were significantly more aggressive with RE of 0.90 and 0.91 respectively. Among the *Fusarium* spp. and in comparison with *V. dahliae*, the *F. sporotrichioides* isolate Fsporo10MN-01 and *V. dahliae* VCG4A isolate VdUSA10-414 did not significantly differ in their RE ($p \leq 0.05$) , although the RE was higher for *V. dahliae* VCG4A isolate VdUSA10-414 than *F. sporotrichioides* isolate Fsporo10MN-01. In addition, there were no significant differences in RE ($p \leq 0.05$) among the four isolates of *Fusarium* spp., *V. dahliae* VCG1 (VdUSA10-18), VCG6 (VdUSA10-17) and VCG2A (VdUSA10-16).

Discussion

Eight different *Fusarium* species causing stem infection on sunflower were identified in this study. While all species are known to exist in the Northern Great Plains states, to the best of our knowledge this is the first report of *F. proliferatum*, *F. culmorum*, *F. avenaceum*, and *F. equiseti* causing disease in sunflower stems in the U.S., and the first report of *F. graminearum* causing stem disease on sunflower in any country. The differences in aggressiveness detected among *Fusarium* species suggest that *F. sporotrichioides* and *F. equiseti* could have a more important role than all other species detected.

Fusarium sporotrichioides was determined to be the most aggressive among the *Fusarium* spp., which is consistent with previous research by Antonova *et al*. (2002). While distribution of the species in sunflower production was beyond the scope of this study, *F. sporotrichioides* was most frequently recovered pathogens from stems in addition to *F. acuminatum*, further suggesting

they may play a more important role than all other species found in the study. However, the most aggressive *Fusarium* isolates were only as aggressive as the least aggressive *V. dahliae* isolates when tested using a root-dip inoculation method. While it is possible that the *Fusarium* species in this study were simply less aggressive than *V. dahliae,* it is also possible that the inoculation method used to compare them is better suited for infection by *V. dahliae* than for evaluation of Fusarium spp. such as *F. sporotrichioides* and *F. equiseti*. The root-dip method has been used to evaluate Fusarium wilt caused by *F. oxysporum*, in crops such as watermelons and dry edible peas (Egel and Martyn, 2007, Kraft and Haglund, 1978). Antonova *et al*. (2002) compared sowing the sunflower seeds directly in artificially infested soil and injection of spore suspension into hypocotyls to study the pathogenicity of *Fusarium* spp. However, both these inoculation methods failed to distinguish resistant and susceptible sunflower genotypes (Antonova *et al*., 2002). Given that choice of inoculation method may have an impact on the infection by *Fusarium* spp. (Kraft and Haglund, 1978), the root-dip method may not be effective in evaluating Fusarium wilt on sunflowers and in particular, the capability of *F. sporotrichioides* and *F. equiseti* as wilt pathogens.

The impact of the *Fusarium* species on sunflower yield in the Northern Great Plains and Europe is unclear. While yield loss due to *Fusarium* has been documented (Gontcharov *et al*., 2006, Aćimović, 1989), the level of yield reductions is difficult to estimate. *Fusarium* species are facultative plant pathogens (Leslie and Summerell, 2006), and infection can be facilitated and/or exacerbated by stress (abiotic or biotic), even if *Fusarium* spp. are not the primary etiological agent. In this study, multiple species were obtained from infected stalks that expressed symptoms consistent with any stem or wilt disease during the survey. Consequently, it is impossible to know if the *Fusarium* species obtained were the primary pathogen causing the symptoms. However, because external symptoms of infection by *Fusarium* is relatively non-specific (Davis *et al*., 2006),

it is also possible that unrecognized yield loss on sunflowers may be occurring throughout the region. It may be prudent to assess both the impact on sunflower yield and distribution of the most aggressive species in the future.

The epidemiological impact of sunflowers being infected by the *Fusarium* species identified in the Northern Great Plains may be important to other crops. Some of these *Fusarium* species are pathogenic on crops used in rotation with sunflowers in the Northern Great Plains states, such as dry edible beans (*Phaseolus vulgaris* L., Bilgi *et al.*, 2008), dry edible peas (*Pisum sativum* L., Mathew *et al.*, 2008), soybeans (*Glycine max* L., Mathew, unpublished), and wheat (McMullen *et al.*, 2008). *Fusarium equiseti* and *F. sporotrichioides* were the most aggressive species identified in this study and are known root rot pathogens of pulse crops (Mathew *et al.*, 2008). As such, the impact of *Fusarium* when the two crops are in a rotation should be considered. Thus, increasing awareness among growers that *Fusarium* can cause disease on sunflowers can aid in monitoring disease prevalence and analysis of crop rotation strategies. Conversely, *F. graminearum*, the primary causal agent of Fusarium head blight (FHB) of cereals, was the least aggressive of all species identified in this study. This is in agreement with other studies (Pereyra and Dill-Macky, 2008, McMullen *et al*., 2008), suggesting sunflower is a good rotational crop for management of FHB for reducing the inoculum level.

The results of this study also demonstrate the potential usefulness of Rep-PCR as an alternate strategy for *Fusarium* identification. The Rep-PCR DNA fingerprint pattern revealed that *Fusarium* isolates from sunflowers possibly belonged to eight species (approximately 75 to 100% similarity, Fig 4.1) and was supported by EF1-α phylogeny (> 95% PP) and morphological identification. However, a second cluster of *F. sporotrichioides* was observed in the Rep-PCR dendogram which did not contain the reference isolate; this could have been as a result of

misidentification of *F. sporotrichioides* using traditional taxonomy and those isolates have to been identified using EF1-α sequencing. While Rep-PCR would not be a replacement for the accuracy needed in identification of *Fusarium* species surveys, it could be particularly useful for routine disease identification, such as that performed by plant disease diagnosticians. While EF1-α provided a more robust resolution of the various *Fusarium* species on sunflower, Rep-PCR is a more simple and convenient PCR-based technique that uses universal primers directed to multiple repetitive DNA sequences and can tolerate a wider range of DNA concentrations in generating reproducible results (Versalovic *et al*., 1994). Accurate morphological identification of *Fusarium* species requires a relatively high degree of mycological experience, while Rep-PCR can be performed with basic molecular knowledge.

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APPENDIX. MEDIAN, MEAN RANK AND RELATIVE TREATMENT EFFECTS ($p \leq 0.05$) FOR SEVERITY RATING OF PHOMOPSIS STEM CANKER ON SUNFLOWER CV. 'HA 288' CAUSED BY THE ISOLATES OF THE TWO *DIAPORTHE* SPECIES

Species	Isolate	Location, Year	Median disease rating			Mean rank			Estimated relative effect (RE)[b]		
			3-d[a]	10-d[a]	14-d[a]	3-d	10-d	14-d	3-d	10-d	14-d
Control			0.0	0.0	0.0	18.5	18.5	18.5	0.05 (0.05,0.05)	0.05 (0.05,0.05)	0.05 (0.05,0.05)
D. gulyae	DG1	SD, 2010	3.0	3.0	3.0	177.7	220.0	220.0	0.44 (0.32, 0.57)	0.55 (0.53,0.57)	0.55 (0.53,0.57)
	DG2	SD, 2010	2.0	3.0	3.0	135.3	220.0	220.0	0.34 (0.22, 0.48)	0.55 (0.53,0.57)	0.55 (0.53,0.57)
	DG3	SD, 2010	2.0	2.5	3.0	93.0	177.3	219.2	0.23 (0.21, 0.25)	0.44 (0.26,0.64)	0.55 (0.33,0.75)
	DG4	SD, 2010	2.0	3.0	3.5	114.2	240.3	282.3	0.28 (0.19, 0.40)	0.60 (0.41,0.77)	0.71 (0.56,0.82)
	DG5	SD, 2010	2.0	2.0	2.0	114.2	135.3	135.3	0.28 (0.19, 0.39)	0.34 (0.22,0.47)	0.34 (0.22,0.47)
	DG6	SD, 2010	3.0	3.0	3.0	240.8	240.8	240.8	0.60 (0.50, 0.70)	0.60 (0.50,0.70)	0.60 (0.50,0.70)
	DG7	SD, 2010	3.0	3.0	3.0	177.7	240.8	240.8	0.44 (0.32, 0.57)	0.60 (0.50,0.69)	0.60 (0.50,0.69)
	DG8	SD, 2010	4.0	4.0	4.0	323.8	344.5	344.5	0.81 (0.69, 0.89)	0.86 (0.85,0.88)	0.86 (0.85,0.88)

[a] Stem lesion size evaluated at 14-d after inoculation using a 0 to 5 scale modified from Thompson *et al*. (2011), where; 0 = no discoloration; 1 = low level discoloration at site of inoculation; 2 = slight discoloration or lesion 1–2 mm in length; 3 = necrotic lesions 2–5 mm in length, some colored stem streaking, leaf wilting and twisting; 4 = lesions 5–10 mm in length, significant necrosis and dark colored stem streaking, leaf and plant wilting, twisting, stunting, and some lodging; 5 = lesions exceeding 10 mm in length, severe leaf necrosis, and wilting, stunting, lodging or plant death.

[b] Relative treatment effects were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004).

Species	Isolate	Location, Year	Median disease rating			Mean rank			Estimated relative effect (RE)[b]		
			3-d[a]	10-d[a]	14-d[a]	3-d	10-d	14-d	3-d	10-d	14-d
D. gulyae	DG9	SD, 2010	2.0	3.0	3.0	135.3	220.0	220.0	0.34 (0.22, 0.48)	0.55 (0.53,0.57)	0.55 (0.53,0.57)
	DG10	SD, 2010	3.0	3.0	3.0	177.7	220.0	220.0	0.44 (0.32, 0.57)	0.55 (0.53,0.57)	0.55 (0.53,0.57)
D. helianthi	DH1	MN, 2011	2.0	2.5	4.0	93.0	156.5	344.5	0.23 (0.21, 0.25)	0.39 (0.26,0.53)	0.86 (0.85,0.88)
	DH2	SD, 2011	2.0	2.0	4.0	93.0	93.0	344.5	0.23 (0.21, 0.25)	0.23 (0.21,0.25)	0.86 (0.85,0.88)
	DH3	MN, 2011	2.0	2.0	2.0	74.5	93.0	93.0	0.18 (0.13, 0.25)	0.23 (0.21,0.25)	0.23 (0.21,0.25)
	DH4	MN, 2011	2.0	2.0	2.5	93.0	135.3	177.2	0.23 (0.21, 0.25)	0.34 (0.22,0.47)	0.44 (0.26,0.64)
	DH5	MN, 2011	3.0	3.5	4.0	177.7	282.3	344.5	0.44 (0.32, 0.58)	0.71 (0.55,0.82)	0.86 (0.85,0.88)
	DH6	MN, 2011	2.0	3.0	4.0	135.3	261.5	344.5	0.34 (0.22, 0.47)	0.65 (0.52,0.77)	0.86 (0.85,0.88)
	DH7	MN, 2011	3.0	4.0	4.0	198.8	323.8	344.5	0.50 (0.39, 0.60)	0.81 (0.69,0.89)	0.86 (0.85,0.88)
	DH8	ND, 2010	3.0	4.0	4.0	198.4	344.5	344.5	0.50 (0.32, 0.68)	0.86 (0.85,0.88)	0.86 (0.85,0.88)
	DH9	MN, 2011	3.0	4.0	4.0	220.0	344.5	344.5	0.55 (0.53, 0.57)	0.86 (0.85,0.88)	0.86 (0.85,0.88)
	DH10	MN, 2011	2.0	3.0	4.0	93.0	261.5	344.5	0.23 (0.21, 0.25)	0.65 (0.52,0.77)	0.86 (0.85,0.88)

[a] Stem lesion size evaluated at 14-d after inoculation using a 0 to 5 scale modified from Thompson *et al*. (2011), where; 0 = no discoloration; 1 = low level discoloration at site of inoculation; 2 = slight discoloration or lesion 1–2 mm in length; 3 = necrotic lesions 2–5 mm in length, some colored stem streaking, leaf wilting and twisting; 4 = lesions 5–10 mm in length, significant necrosis and dark colored stem streaking, leaf and plant wilting, twisting, stunting, and some lodging; 5 = lesions exceeding 10 mm in length, severe leaf necrosis, and wilting, stunting, lodging or plant death.

[b] Relative treatment effects were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004).