DOWNY MILDEW OF SUNFLOWERS: ESTABLISHMENT OF BASELINE SENSITIVITY TO AZOXYSTROBIN AND MONITORING FOR THE DEVELOPMENT OF FUNGICIDE RESISTANCE AND *PLASMOPARA HALSTEDII* VIRULENCE PHENOTYPE CHANGES

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Downy mildew of sunflowers: Establishment of baseline sensitivity to azoxystrobin and monitoring for the development of fungicide resistance and *Plasmopara halstedii* virulence phenotype changes

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ABSTRACT

Downy mildew, caused by *Plasmopara halstedii* (Farl.) Berl. and de Toni, is an economically important disease in cultivated sunflowers, *Helianthus annuus* L. The objectives of this study were to determine disease pressure in North Dakota and South Dakota, determine the virulence phenotypes in the pathogen population, determine the baseline sensitivity to azoxystrobin and evaluate select isolates for fungicide insensitivity. While downy mildew was present in many fields, incidence was typically low. To determine virulence phenotypes, selected isolates were evaluated on an expanded set of differential lines. New virulence was found to the Pl_8 resistance gene, but no virulence was observed on the Pl_{Arg}, Pl_{15}, Pl_{17} and Pl_{18} genes. Using a discriminatory dose of 10 ug ai azoxystrobin/seed, no isolate approached infection levels found in inoculated, nontreated controls; therefore, the pathogen is considered sensitive to azoxystrobin in the greenhouse and azoxystrobin should still suppress downy mildew in the field.

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Introduction: Importance

Downy mildew, caused by the biotrophic oomycete pathogen *Plasmopara halstedii* (Farl.) Berl. and de Toni, is an economically important disease in cultivated sunflowers, *Helianthus annuus* L. Downy mildew occurs sporadically within a field, and is dependent on free-soil moisture and cool temperatures following planting (Zimmer 1975). The first major U.S. outbreak of the disease occurred in 1970 in the Red River Valley of North Dakota and Minnesota, where incidence was as high as 90% in some fields (Kolte 1985). Since then, downy mildew has been frequently reported in the region. Sunflower fields have been surveyed since 2001 for the prevalence and incidence of downy mildew as part of the North Dakota State University-Integrated Pest Management (NDSU-IPM) survey and the National Sunflower Association (NSA) Crop Survey (Gulya et al. 2013). Yearly prevalence for the NDSU-IPM survey between 2001 and 2011 ranged from 1.6% to 70.3% in North Dakota and averaged 33.9% based on an average of 141 fields per year (Gulya et al. 2013). Yearly prevalence for the NSA survey between 2001 and 2011 ranged from 1.5% to 47.5% in North Dakota and averaged 17.0% based on an average of 121 fields per year (Gulya et al. 2013). The years with the highest prevalence for the NDSU-IPM survey were 2004 (58.5%), 2005 (58.8%) and 2011 (70.3%), while similarly 2005 (47.5%) and 2011 (40.2%) had the highest prevalence in the NSA survey (Gulya et al. 2013). The NSA survey was not conducted in 2004 and became a bi-annual survey in 2013. The NDSU-IPM survey indicated that 12% of 162 fields in 2012, 38% of 94 fields in 2013, 54% of 191 fields in 2014 and 31% of 235 fields in 2015 had some level of downy mildew (Gulya et al. 2014; Knodel 2014; 2015). The NSA survey identified downy mildew in 11% of

97 fields in 2012, 9% of 100 fields in 2013 and 25.5% of 55 fields in 2015 in North Dakota (H. Kandel, *unpublished data 2012*; Kandel 2014; Kandel and Gulya 2016).

Most infected plants die and those that survive produce smaller, lighter seeds with lower oil content and compete with healthy plants for resources (Friskop et al. 2009; Zimmer and Zimmerman 1972). Yield losses due to downy mildew are dependent on the number of systemically infected plants and their distribution within the field (Friskop et al. 2009; Zimmer 1975). Often, many infected plants are found clustered together which can cause substantial yield losses (Friskop et al. 2009). Yield loss from scattered infected plants can be offset by adjacent healthy plants (Friskop et al. 2009). With scattered infection, incidence below 15% should result in minimal yield loss (Bradley et al 2007; Gulya et al. 2013).

Sunflower

Origin and History

Sunflowers are of North American origin (Heiser 1951; Leppik 1966). Cultivated sunflower, *H. annuus*, is one of 14 annual and 39 perennial *Helianthus* species native to the Americas and is in the Asteraceae family Asteroideae subfamily Heliantheae tribe (Sackston 1992; Schilling, Flora of North America 1993+; G. Seiler, *personal communication*). Sunflowers were first domesticated by Native American populations, where they were selected from a branched annual into a single headed plant with larger achenes and were used for food, oil, dye and medicine (Harveson 2016; Heiser 1951; Rogers et al. 1982). Wild annual sunflowers are believed to have been domesticated prior to 2300 BCE according to radiocarbon dated achenes found in Tennessee (Crites 1993; Smith 2006) and in Mexico (Lentz et al. 2008). Spanish explorers introduced sunflowers to Europe as an ornamental flower in the 16th century

(Heiser 1998). In the 19th century, Russian breeders developed the two modern types of cultivated sunflowers: oilseed and non-oilseed (confection) (Gulya et al. 1997).

Sunflower Production in the United States

Sunflower germplasm from Russia is believed to have been brought to the United States from multiple sources after 1875, reintroducing sunflowers as a field crop (Putt 1997). From 1900 to 1940, sunflowers were grown for silage and poultry scratch feeds (Putt 1997). Commercial production of confection seed started in the mid-1960's and peaked in 1974 at 97,000 hectares (Putt 1997). Commercial production of oilseed sunflower became significant in the United States in 1966 due to the introduction from Russia of cultivars with increased oil content (Berglund 2007; Putt 1997). The combined discoveries of cytoplasmic male sterility by Leclerq (1968) in France and fertility restoring genes in 1970 in Canada and the United States enabled production of hybrid sunflower seed (Enns et al. 1970; Kinman 1970; Putt 1997). Commercial sunflower production peaked in the United States at over 2.2M hectares in 1979 after open-pollinated cultivars were replaced by hybrids (Berglund 2007; Putt 1997). Currently in the United States, sunflowers are primarily grown in the plains states of North Dakota, Minnesota, South Dakota, Nebraska, Colorado, Kansas, Oklahoma and Texas. Approximately 730,000 hectares of sunflowers were harvested in 2015 with North Dakota and South Dakota planting 70% of the total hectarage (NASS 2016). Oil hybrids comprise 84% of the crop hectarage in the United States and are grown primarily for frying oil and the sunflower meal by-product used for animal feed (NASS 2016). The confection hybrids typically have much larger heads and seeds and are used for direct human consumption. Both sunflower types are used for bird seed.

Plasmopara halstedii – Downy Mildew

Taxonomy

Plasmopara halstedii is currently classified as an oomycete in the Stramenopile group of the eukaryotes in the Peronosporales order and Peronosporaceae family. *Plasmopara halstedii* originated in North America (Kolte 1985; Novotelnova 1966). The pathogen was first described as *Peronospora halstedii* by Dr. W. G. Farlow in 1883 in the Proceedings of the American Academy of Arts and Sciences on a *Eupatorium purpureum* sample collected by B. D. Halsted, a graduate student in cryptogamic botany (Stevens et al. 1920; Virányi and Spring 2011). The sample was collected in 1876 in Massachusetts near Harvard University's Bussey Institution (Stevens et al. 1920; Virányi and Spring 2011). After *Plasmopara* was separated from *Peronospora*, Berlese and de Toni transferred the taxon in 1888 to the new *Plasmopara* genus (Virányi and Spring 2011). The first documented cases of *P. halstedii* on *H. annuus* were in the 1890's (Virányi and Spring 2011). By 1924, downy mildew on *H. annuus* had been reported in the states of Indiana, Iowa, Minnesota, Montana and New York (Henry and Gilbert 1924; Young and Morris 1927). Sunflower downy mildew has been identified in all regions where sunflowers are grown with the exception of Australia and New Zealand (CABI 2016; Constantinescu and Thines 2010).

Host Range

Downy mildew is found in both cultivated and wild species of *Helianthus* (Bradley et al. 2007). Sunflower downy mildew is assumed to have spread internationally primarily through contaminated seed (CABI 2016; Kolte 1985; Leppik 1966). *Iva annua* L., marsh elder, and other weeds in the Asteraceae family may also act as reservoirs of inoculum (Bradley et al. 2007). *Plasmopara halstedii* also causes downy mildew on other genera in the Heliantheae tribe

including *Ambrosia* spp., *Bidens* spp., *Clibadium asperum*, *Franseria discolor*, *Hemizonia luzulaefolia*, *Madia dissitiflora*, *Ratibida pinnata*, *Rudbeckia* spp., *Silphium spp.*, *Spilanthes americana*, *Verbesina* spp. and *Zinnia* sp. as well as other genera in other tribes in the Asteraceae family (Farr and Rossman 2017).

Life Cycle

Plasmopara halstedii is an obligate biotroph; therefore, it requires a living sunflower plant to complete its life cycle. Sexual, thick walled oospores with diploid nuclei germinate to form zoosporangia (Friskop 2009). These zoosporangia release a few to a dozen haploid asexual, motile, biflagellate zoospores (Humann et al. 2016; Spring et al. 1998). Within 24 hours after contacting sunflower radicles, most zoospores lose the flagella and encyst (Gascuel et al. 2015). Usually, they then form a germ tube and apressorium which penetrates the root directly. However, zoospores can also enter through wounds frequently present at the base of root hairs or directly through the epidermal cell wall (Gascuel et al. 2015). After penetration, one to three or more haustoria are produced per plant cell for nutrients (Novotelnova 1966). Parasexual recombination of genetic material without gametangiogamy leading to increased genetic diversity is believed to occur at the zoospore stage of the life cycle, but the mechanism has not been determined (Spring and Zipper 2006; 2016). Once inside the root, the pathogen progresses as hyphae, grows up the stem and throughout the intercellular places of the plant (Spring 2000). During periods of cool temperatures between 10 and 15℃ and high relative humidity, asexual sporulation will occur (Baldini et al. 2008). Branched dissemination structures called zoosporangiophores, bearing up to 20 zoosporangia, will emerge from stomata primarily on the underside of the cotyledons and true leaves (Gascuel et al. 2015). Sexual oospores are produced homothallically at mycelial tips under the epidermis when a fertilization tube from the

antheridium enters the oosphere in the oogonium (Kolte 1985; Spring 2000). The oospore develops a thick wall enabling *P. halstedii* to overwinter for up to ten years in the soil (Spring 2000).

Symptoms and Signs

Symptoms of sunflower downy mildew infections vary depending on whether they are primary, caused by an infection of the roots by oospores, or secondary, caused by an infection of the aboveground parts by airborne zoosporangia from infected plants (Meliala et al. 2000). Primary systemic infection of seedling roots may cause damping-off before or after germination (Gulya et al. 1997). If seedlings survive, cotyledons and the first true leaves become thickened, puckered and chlorotic (Gulya et al. 1997). Initially, leaves show chlorosis only along the veins, but over time this can extend across the leaf. Periods of dew and cool temperatures will facilitate sporulation on the underside of the leaves, where the signs of the pathogen, mycelia and zoosporangia, appear on the underside of the chlorotic areas. Plants that survive into reproductive stages are severely dwarfed with shortened internodes and horizontal heads.

Secondary infection in the field may occur when windblown zoospores from systemically infected plants land on sunflower leaves or flower buds (Meliala et al. 2000). If the environment is favorable for infection, small, angular and chlorotic lesions will form on the upperside of the leaf. In favorable conditions, mycelia and zoosporangia will form immediately opposite the lesion on the underside of the leaf (Gulya et al. 1997). These localized lesions may turn necrotic and typically remain bounded by veins. However, in some cases, the pathogen may enter a vein and start moving up the plant towards the apical meristem (Spring 2009). Secondary infection occurring when sunflower plants have only cotyledons or one pair of true leaves would look similar to primary systemic infections caused by root infection (Meliala et al. 2000; Spring

2009). Plants infected later could express a delayed systemic reaction with stunting only above the infection site (Meliala et al. 2000). Field conditions causing yield limiting secondary infections are rare in the United States (Friskop et al. 2009).

Epidemiology

The impact of downy mildew on yield in the North Central Great Plains is dependent on the timing of rain after planting and inoculum. If inoculum is present, level ground, low spots, soil with high clay content or anything which keeps the soil water from draining will increase yield losses due to large areas being affected. Sunflower downy mildew epidemics are more likely when high levels of inoculum are present in the soil or when zoosporangia from nearby cultivated, volunteer or wild sunflowers blow onto young plants or spores flow onto or through the field in irrigation water or runoff (Gulya et al. 1997; Zimmer 1972). Seed-borne transmission is possible, but is unlikely to infect many plants (Gulya et al. 1997).

Cultivated sunflower seedlings are most susceptible to systemic infection by *P. halstedii* from germination to emergence, when seedling roots are shorter than 5 cm long (Bradley et al. 2007). Infection is most likely to occur during the five days after germination, but can occur up to the four-leaf stage (V-4) two to three weeks after germination (Cohen and Sackston 1973; Gascuel et al. 2015; Schneiter and Miller 1981; Zimmer 1975). The longer it takes for seedlings to emerge, the more likely they are to get infected. Germination speed is heavily dependent on soil temperature and moisture. Zoospores germinate between 4 and 22ºC and can infect plants between 6 and 26ºC (Novotelnova 1966). Ideal temperatures for the pathogen have been reported to be an average air temperature between 10 and 15°C in the five days after planting (Baldini et al. 2008). Since *P. halstedii* requires high soil moisture for zoospore movement and

root infection, rain or irrigation three to fifteen days after planting will increase the chance of infection (Gulya et al. 1997).

Sunflower plants remain susceptible to secondary infection on leaves and buds for their entire lifespan (Spring 2009). Little information is available on the mechanisms of development of local lesions and their transition to systemic infections along the veins (Gascuel et al. 2015).

Management of Downy Mildew

Planting downy mildew resistant hybrids is one of the most important tools for sunflower downy mildew management; however, many previously deployed, single, dominant resistance genes (denoted *Pl*) have been overcome by the pathogen (Tourvieille de Labrouhe et al. 2008). Fungicidal seed treatments are widely used, but the pathogen has also overcome at least one chemical mode of action. Cultural management practices such as crop rotation, avoiding poorly drained parts of fields and removing wild and volunteer sunflowers can reduce the inoculum to help manage the downy mildew problem in sunflowers.

Genetic Resistance

Resistance to downy mildew in sunflowers has been qualitative through the use of single dominant resistance genes denoted *Pl (for Plasmopara)* (Tourvieille de Labrouhe et al. 2008). Single gene resistance can be overcome quickly, if the same single resistance gene is used consecutively (Tourvieille de Labrouhe et al. 2010). Pyramiding single resistance genes or rotating hybrids with different *Pl* genes in the field would make resistance to downy mildew more durable (Tourvieille de Labrouhe et al. 2008; 2010). However, seed companies do not label their seed with the *Pl* resistance gene used, making gene rotation by growers nearly impossible. Pyramiding multiple resistance genes is difficult due to the time and resources required using classical breeding techniques and the limited molecular markers available. Many

previously deployed resistance genes have been overcome by the pathogen (Tourvieille de Labrouhe et al. 2008). Consequently, periodic monitoring of pathogen changes remains important for breeding and selecting resistant hybrids.

Germplasm from Russia was re-introduced to the United States without downy mildew resistance. The first oilseed downy mildew resistance was an unintended result of wild *H. annuus* crosses in Texas between 1949 and 1953 with Canadian lines CM953-102 and CM953-88 (Slabaugh et al. 2003). The subsequent germplasm provided rust resistance and linked downy mildew resistance (Slabaugh et al. 2003). This germplasm was later used in the development of the USDA oilseed restorer line and maintainer line germplasm (Slabaugh et al. 2003; Vear et al. 2008a). The linked rust and downy mildew resistance was believed to be introduced into confectionary lines RHA 280, RHA 282 and HA 287 from accidental crossing of a few plants of the cultivar 'Commander' (J. Miller*, personal communication*, as cited in Slabaugh et al. 2003).

Beginning in the 1960's, genetic resistance identified in cultivated oilseed sunflowers was introgressed into inbred lines (Vear et al. 2008a). Maintainer line HA60 was released in 1968 and restorer lines RHA 265 and RHA 266 were released in 1971 with Canadian line CM953-102 containing resistance gene Pl_1 as part of their pedigree (Sunflower inbreds 2006b; Vear et al. 2008a). Restorer line germplasm RHA 271, RHA 273 and RHA 274 were released in 1973, all with resistance gene Pl_2 from HA 62, a sister selection to maintainer line HA 61 which was Canadian line CM953-88 and is believed to provide the downy mildew resistance (Sunflower inbreds 2006a; Vear et al. 2008a). While RHA 274 also contained the resistance gene Pl_1, four other genes (Pl_9, Pl_{10}, Pl_{11} and Pl_{12}) were also identified in the line later (Gulya et al. 1991b; Liu et al. 2012; Molinero-Ruiz et al. 2003; Rahim et al. 2002). These first resistance

genes deployed were effective in North America until the pathogen developed virulence to all of them by 1980 (Carson 1981; Miller and Gulya 1984).

In the 1980's, an increased effort to identify downy mildew resistance from wild *Helianthus* germplasm began. DM-2, a composite of plants originating from the open-pollinated accession Novinka with resistance from *H. tuberosus*, was released in 1984 with resistance genes Pl_5, Pl_{11} and Pl_{12} (Miller and Gulya 1984; Liu et al. 2012; Rahim et al. 2002; Vear et al. 2008a). HA-R4 and HA-R5 were also released in 1984 and contained resistance genes Pl_{16} and Pl_{13}, respectively, both derived from cultivated sunflowers (Gulya 1985; Liu et al. 2012; Mulpuri et al. 2009; Vear et al. 2008a). Downy mildew resistance in HA-R4 is believed to be derived from an Argentinean pool with Russian open pollinated cultivars crossed with *H. annuus*, *H. argophyllus* and *H. petiolaris* in 1955 and 1956 (González et al. 2015; Vear et al. 2008a). Downy mildew resistance in HA-R5 is believed to come in part from Canadian line 953-102 (González et al. 2015; Vear et al. 2008a). In 1986, the USDA released six downy mildew resistant lines: Pl_6 in HA 335 and HA 336 from wild *H. annuus*, Pl_7 in HA 337, HA 338 and HA 339 from *H. praecox* and Pl_8 in RHA 340 from *H. argophyllus* (Miller and Gulya 1991).

During the 1990's, some of the new downy mildew resistant lines, including those containing resistance genes Pl_5, Pl_6, Pl_7 and Pl_8, were used by seed companies to develop commercial hybrids (Vear et al. 2008b). Two downy mildew resistant lines were released in 1999 by USDA from *H. argophyllus***,** RHA 419 and RHA 420 (Miller et al. 2002). The gene in these two hybrids was called Pl_{Arg}, breaking from the traditional numbering of resistance genes even though it was not the first resistance gene found from *H. argophyllus* (DuBle et al. 2004; Imerovski et al. 2014; Vear et al. 2008a).

Several additional lines with new downy mildew resistance genes have been released since 2000, including HA 458 released in 2006 with gene Pl_{17} from wild *H. annuus*, HA DM 1 released in 2015 with gene Pl_{18} from *H. argophyllus*, and RNID, a proprietary inbred line from Nidera, S.A. in Argentina, with resistance gene Pl_{15} (Bertero de Romamo et al. 2010; DuBle et al. 2004; Paniego et al. 2012; Qi et al. 2015; 2016; Vear et al. 2008a). In 2001, RHA 436, RHA 437 and RHA 438 were released with Pl_8 from RHA 340 and high oleic germplasm (Miller et al. 2004). In 2006, RHA 468 was released with Pl_{Arg} from RHA 419 (B. Hulke, *personal communication*). In 2008, HA 460, a maintainer line, was released with Pl_8 from RHA 340 and high oleic germplasm (Hulke et al. 2010). Pl_{19} from wild *H. annuus* has been introgressed into confection sunflower and it is hoped that Pl_{19} will be combined with Pl_{Arg} and Pl_{18} into a single line for durable downy mildew control in confection sunflowers (Zhang et al. 2016).

Virulence Phenotypes

Plasmopara halstedii has quickly been able to adapt to, and overcome, many resistance genes that have been deployed. This is due at least in part to the highly variable nature of the pathogen, but also to the large number of major genes that have been deployed singly in a global production system (Markell et al. 2016; Virányi et al. 2015). *Plasmopara halstedii* virulence to resistance genes Pl_1 and Pl_2 led to the development of the first two virulence phenotypes of *P. halstedii* (Sackston 1981). The first two virulence phenotypes were separated geographically, so the "European" race and the "North American" race (syn: "Red River" race) were easily distinguished (Gulya et al. 1997). As the number of resistance genes and virulence phenotypes increased, a nomenclature system was needed to distinguish virulence phenotypes. Initially, a simple numbering system was used in the United States, where the "European" race became known as "Race 1" and the "North American" race became known as "Race 2" (Gulya et al.

1997). "Race 3" was recognized in the United States in 1980 and by 1990, the United States named "Races 4 and 5" (Carson 1981; Sackston et al. 1990). Simultaneously, France had developed a different race nomenclature, where letters were used to denote different virulence combinations. As the number of "races" increased in both countries, the two different nomenclatures impeded communication and the deployment of effective resistance internationally (Gulya et al. 1998). In an attempt to address this, a 1990 proposal was made by Canada and the United States to develop a nomenclature using a gene based system to compare virulence phenotypes between countries (Sackston et al. 1990). In 1991, the proposed gene based virulence formulas for the first eight races were published, with Race 8, for example, becoming known as Race 1,2,3,4,a,b (Gulya et al. 1991a). However, this was not adopted because it was deemed cumbersome and because some of the *Pl* genes are actually clusters of genes which provide resistance to one or more races (Gulya et al. 1998; Vear et al. 1997).

In 1998, an international proposal at the International Sunflower Association Symposium on Sunflower Downy Mildew in Fargo, North Dakota identified nine standardized, publicly available, fixed inbred lines with consistent downy mildew reactions and different resistance genes or gene combinations that could be used as differential lines, and when used in three sets of three, an easy, concise race nomenclature system (Gulya et al. 1998). The following differential lines were proposed: HA 304, RHA 265, RHA 274, PM13, PM-17, 803-1, HA-R4, QHP1 and HA 335 (Table 1) (Gulya et al. 1998). New differentials were to be added as needed in sets of three (Gulya et al. 1998).

In 2000, after testing the existing races of downy mildew on the proposed standardized set of nine differential lines, ten races were described and the new nomenclature was presented at the International Sunflower Conference in Toulouse, France (Tourvieille de Labrouhe et al.

Table 1. Internationally accepted differential set of sunflower lines used for determination of virulence phenotype for races of *Plasmopara halstedii* and known resistance gene.

Differential Set [a]		Resistance Gene[b]
D-1	HA 304	None
D-2	RHA 265	Pl_1
D-3	RHA 274	Pl_2/Pl_{21}
D-4	PMI3	Pl_5
D-5	PM-17	Unknown
D-6	803-1	Unknown
D-7	HA-R4	Pl_{16}
D-8	QHP1	Pl_{13}
D-9	HA 335	Pl_6

[a]D-1 to D-9, lines of differential set.
[b]*Pl* indicates *Plasmopara* resistance gene where known (Liu et al. 2012; Miller and Gulya 1991; Molinero-Ruiz et al. 2003; Mulpuri et al. 2009; Rahim et al. 2002; Vincourt et al. 2012; Zimmer and Kinman 1972).

2000). After confirming the reactions of the lines, it was also proposed that "susceptible" meant sporulation had to occur on the true leaves (Tourvieille de Labrouhe et al. 2000). Only differential lines 3 (RHA 274), 8 (HA-R4) and 9 (HA 335) have consistently been accepted by scientists as the standard line (Trojanová et al. 2017). The differential line and the gene associated with it cannot be changed without destroying the continuity of races, aggregate virulence phenotypes. Because some of the sunflower lines selected can either be difficult to grow or to rate for virulence the differential lines are regularly discussed and changed. In the United States, Dr. Gulya substituted a suitable hybrid for differential line 1, the susceptible check, and used DM-2 instead of PMI3, an INRA selection of USDA composite DM-2, for Pl_5 and HA-R5 instead of QHP1, an INRA cross with HA-R5, for Pl_{13} (T. Gulya, *personal communication*).

In 2012, Institut National de la Recherche Agronomique (INRA) proposed changes to two of the original nine differentials; GB would replace HA 304, the susceptible check, and QHP2 would replace QHP1 for Pl_{13} (Tourvieille de Labrouhe et al. 2012). Two additional sets of three differentials, Y7Q, PSC8, XA, PSS2RM, VAQ and RHA 419, were proposed to

distinguish additional virulence not represented by genes in the original lines (Tourvieille de Labrouhe et al. 2012). These proposed differential lines have not been internationally endorsed (Trojanová et al. 2017).

The pathogen continues to evolve under selection pressure, enabling it to overcome resistance genes. Currently, 42 races of *P. halstedii* have been found internationally and 23 races have been found in the United States (Virányi et al. 2015). A single isolate virulent on all nine of the internationally accepted differential lines has not yet been found; however, virulence to all nine lines has been found (Gascuel et al. 2015; Virányi et al 2015). Between 2009 and 2013 nine races overcame the Pl_6 gene in the United States (Gulya et al. 2014). As of 2013, virulence on the Pl_8 gene has not yet been found in the United States (Gulya et al. 2014). Virulence on the Pl_{16} and Pl_{13} genes was found in 2.8% of 470 isolates collected in the United States between 1998 and 2009 and was rarely found between 2010 and 2013 (T. Gulya, *personal communication*). Periodic international surveys should be undertaken to monitor development of new virulence phenotypes (races) as well as virulence against new lines with different genetic resistance.

Fungicidal Seed Treatments

Fungicidal seed treatments have been widely used for protection against downy mildew (Gulya et al. 1997). The phenylamide fungicide seed treatments metalaxyl (Allegiance, Gustafson, Plano, TX) and mefenoxam (Apron-XL, Novartis, Greensboro, NC) were very effective at managing downy mildew between 1985, when metalaxyl was granted a federal registration on sunflower, until its widespread failure in 1998 and 1999 (Gulya et al. 1999; Gulya 2000). The first reported insensitivity of *P. halstedii* isolates to metalaxyl was in 1995 in France (Albourie et al. 1998). Insensitivity to these fungicides had already been reported in the

oomycete pathogens *Plasmopara viticola, Bremia lactucae* and *Pseudoperonospora cubensis* causing downy mildew of grapes (*Vitis vinifera* L.), lettuce (*Lactuca sativa*) and cucurbits (Cucurbitaceae), respectively, and late blight (*Phytophthora infestans*) and pink rot (*Phytophthora erythroseptica)* of potatoes (*Solanum tuberosum* L.), so it was not altogether unexpected (Abu-El Samen et al. 2005; Gisi and Sierotzki 2015; Grünwald et al. 2006; Heaney et al. 2000). However, it was hypothesized that the risk of resistance development for metalaxyl and mefonoxam would remain low because *P. halstedii* was a soilborne pathogen and the fungicide was applied only once per year as a seed treatment (Gulya et al. 1999).

Following the discovery of metalaxyl and mefenoxam insensitivity, two additional fungicides were found to have varying levels of efficacy: azoxystrobin and fenamidone (Gulya 2002). Both fungicides are considered to be fungistatic rather than fungicidal at the rates tested (Gulya 2002). Both azoxystrobin and fenamidone are in the FRAC 11 fungicide group, the quinone outside inhibitors (QoIs), and are classified as having a high risk of resistance development (FRAC 2016). Azoxystrobin was labeled for use in the United States in 1997 as Heritage (Syngenta Crop Protection, Greensboro, NC) on turf and Abound (Syngenta Crop Protection, Greensboro, NC) on fruit, nuts and vegetables (Uttley 2011). The first known use of azoxystrobin on farm fields in the states of North Dakota, Minnesota, Nebraska and Wisconsin was after July 30, 1998 on potatoes after an emergency use label was granted (Pasche et al. 2004). Azoxystrobin was labeled for use on sunflowers as Protégé (Gustafson, Plano, TX) in 2003 and later as Dynasty (Syngenta Crop Protection, Greensboro, NC) (Bradley 2003). Fenamidone, first sold in 2001, was released as Idol (Bayer Crop Science, Research Triangle Park, NC) and its label was amended to include sunflowers in October of 2007 (Kish 2007).

Fenamidone was withdrawn in 2012 leaving only azoxystrobin, which was labeled only for suppression of downy mildew of sunflowers.

Azoxystrobin, like the other QoIs, targets respiration in fungal and fungal-like mitochondria by binding to an enzyme on the quinone outside site of the cytochrome bc1 complex, preventing the transfer of electrons between cytochrome b and cytochrome c1 (Fernandez-Ortuño 2008). To date, three mutations in the cytochrome b gene, which differentially effect pathogen sensitivity, have been found. These amino acid substitutions either slow or prevent binding and are considered to be qualitative selection (FRAC 2014). G143A, the most common substitution, is a change from glycine to alanine at position 143 and causes complete failure of the fungicide in the field (FRAC 2014). With the G143A mutation, the resistance factor (RF=EC_{50} of the resistant strain/EC_{50} of the sensitive strain) usually exceeds 100 and can be several hundred (FRAC 2014). F129L, a change from phenylalanine to leucine at position 129, and G137R, a change from glycine to arginine at position 137, cause reduced sensitivity that is still controlled by the fungicide (FRAC 2014). As of 2012, FRAC has confirmed field resistance in 25 pathogens with the G143A mutation, five pathogens with the F129L mutation, two pathogens with both the G143A and the F129L mutations and one pathogen with all three mutations (FRAC 2012).

As in the case of metalaxyl and mefenoxam, azoxystrobin applied as an annual seed treatment has a lower risk of resistance development than azoxystrobin applied multiple times during a season as a foliar fungicide (Russell 2003). However, the related foliar oomycete pathogens, *P. viticola, P. cubensis* and *Pythium aphanidermatum* have developed insensitivity to azoxystrobin (FRAC 2012; Genet et al. 2006; Gisi et al. 2000; Ishii et al. 2001). *Plasmopara viticola* has both the G143A and the F129L mutations, *P. cubensis* has the G143A mutation and

P. aphanidermatum has the F129L mutation (FRAC 2012). A baseline to monitor resistance development to azoxystrobin has not been developed.

Oxathiapiprolin, an oomycete fungicide, has been developed by DuPont (Wilmington, DE) and it is very efficacious for downy mildew (Humann 2016). Oxathiapiprolin has recently been reassigned from FRAC group U15 with an unknown mode of action to FRAC Code 49 with target site, lipid homeostasis and transfer/storage, and code F9 (FRAC 2017). Oxathiopiprolin is believed to inhibit a single site, the oxysterol-binding protein homologue; therefore, its resistance risk is assumed to be medium to high and resistance management strategies should be used (FRAC 2017). Oxathiopiprolin has been labeled as Lumisena (Dupont, Wilmington, DE) and Plenaris (Syngenta Crop Protection, Greensboro, NC) and is expected to be used by sunflower growers in the 2017 growing season.

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CHAPTER 1. ASSESSMENT OF DISTRIBUTION, INCIDENCE AND PREVALENCE OF DOWNY MILDEW IN THE NORTH CENTRAL GREAT PLAINS

Introduction

Disease surveys are commonly conducted on economically important diseases of many crops. The information collected in surveys can determine if a particular disease exists in an area, and if found, how severe the problem is. Repeated monitoring of these disease problems by surveys can generate information that can identify shifts in disease presence and incidence over time. Consequently, disease surveys are commonly the first step in identifying significant changes that impact diseases and disease management, including changes in farming practices, evaluation of new pathogen virulence phenotypes and pathogen resistance to fungicides.

Downy mildew of sunflowers is an economically important disease caused by the biotrophic oomycete pathogen *Plasmopara halstedii* (Farl.) Berl. and de Toni. The impact of downy mildew on yield in the North Central Great Plains is dependent on the timing of rain after planting. Cultivated sunflower seedlings are most susceptible to systemic infection by *P. halstedii* from germination to emergence, when seedling roots are shorter than 5 cm long (Bradley et al. 2007). Infection is most likely to occur during the five days after germination, but can occur up to the four-leaf stage (V-4) two to three weeks after germination (Cohen and Sackston 1973; Gascuel et al. 2015; Schneiter and Miller 1981; Zimmer 1975). In North Dakota, yield losses due to downy mildew are dependent on the number of systemically infected plants and their distribution within the field (Friskop et al. 2009; Zimmer 1975). Often, many infected plants are found clustered together which can cause substantial yield losses (Friskop et al. 2009). Since *P. halstedii* requires high soil moisture for zoospore movement and root infection, level ground, low spots, soil with high clay content or anything which keeps the soil water from

draining will increase yield losses due to large areas being affected. Downy mildew of sunflowers is primarily a seedling disease, so surveying should be conducted while the plants are still small to make it easier to identify downy mildew symptoms before infected plants have died (T. Gulya, *personal communication*).

Sunflower fields have been surveyed since 2001 for the prevalence and incidence of downy mildew as part of the North Dakota State University-Integrated Pest Management (NDSU-IPM) survey and the National Sunflower Association (NSA) Crop Survey (Gulya et al. 2013). For these surveys, prevalence is the percent of fields with downy mildew and incidence is the percent of plants in each field with downy mildew (Gulya et al. 2013). The NDSU-IPM survey obtains information about insects, disease and plant stages between late June and mid-August for multiple crops in North Dakota proportionate to hectares planted in a county. Plant growth for sunflowers is divided into Vegetative (V) or Reproductive (R) development (Schneiter and Miller 1981). Vegetative stages are further subdivided into Vegetative Emergence (VE), from when the hypocotyl and cotyledons break through the soil surface up until the first true leaves are at least 4 cm long where the growth stage is then based on the number of true leaves that are 4 cm long (V-2, V-4, etc.) (Schneiter and Miller 1981). When the inflorescence appears, reproductive growth stages begin with R-1 and go until R-9 (Schneiter and Miller 1981). The NSA's multi-state fall survey collects yield and agronomic data and identifies yield limiting factors including disease, weeds, bird and insect damage, lodging, plant spacing, drought and hail.

Yearly prevalence for the NDSU-IPM survey between 2001 and 2011 ranged from 1.6% to 70.3% in North Dakota and averaged 33.9% based on an average of 141 fields per year (Gulya et al. 2013). Yearly prevalence for the NSA survey between 2001 and 2011 ranged from 1.5% to

47.5% in North Dakota and averaged 17.0% based on an average of 121 fields per year (Gulya et al. 2013). The years with the highest prevalence for the NDSU-IPM survey were 2004 (58.5%), 2005 (58.8%) and 2011 (70.3%), while similarly 2005 (47.5%) and 2011 (40.2%) had the highest prevalence in the NSA survey (Gulya et al. 2013). The NSA survey was not conducted in 2004 and became a bi-annual survey in 2013. The NDSU-IPM survey indicated that 12% of 162 fields in 2012, 38% of 94 fields in 2013, 54% of 191 fields in 2014 and 31% of 235 fields in 2015 had some level of downy mildew (Gulya et al. 2014; Knodel 2014; 2015). The NSA survey identified downy mildew in 11% of 97 fields in 2012, 9% of 100 fields in 2013 and 25.5% of 55 fields in 2015 in North Dakota (H. Kandel, *unpublished data 2012*; Kandel 2014; Kandel and Gulya 2016). Neither sunflower survey was designed solely for downy mildew observations, so plants that died early from downy mildew may no longer have been visible in fields surveyed when the sunflowers were blooming or mature (Gulya et al. 2013). The objective of this two-year study was to assess the distribution, incidence and prevalence of downy mildew in North Dakota and South Dakota in a survey designed specifically to evaluate downy mildew in the geographic areas with the most sunflower fields.

Materials and Methods

The two-year survey was conducted between June 30 and July 10 of 2014 and between July 8 and July 24 of 2015 when host growth stage and environmental conditions made it most likely that downy mildew symptoms would be most visible. To develop a survey route of sunflower fields, CropScape and Cropland Data Layers (USDA 2014) were used to identify current high-density sunflower growing areas in North Dakota and South Dakota. Individual fields were arbitrarily selected and were typically at least 3 to 8 km apart. Additionally, plant

growth stages varied among surveyed fields since the timing of rainfall after planting is so critical to disease development.

To determine field incidence expressed as a percent, a visual inspection was made for downy mildew signs and symptoms on 40 plants at five points in an inverted W-shaped pattern for a total of 200 plants. Symptoms and signs of downy mildew included dead or dying plants, stunting, chlorotic leaves and zoosporangia on the underside of the leaves in the chlorotic areas (Friskop 2009). Incidence was determined for each field surveyed and is defined as the percentage of plants infected out of 200 plants. Occurrence is defined as having observed downy mildew in a field in trace quantities where the disease was present, but was not observed in the 200 plants used to calculated incidence at the survey points. Prevalence was determined based on whether the disease was present or absent in a field. Downy mildew was considered present if one plant with symptoms was identified anywhere in the field; therefore, a field with zero incidence in the 200 plants assessed was considered infected with downy mildew if a plant was found elsewhere in the field. Prevalence is defined as the percent of fields with downy mildew. Plant growth stages and GPS location were recorded as well as presence of other diseases.

Results

In 2014, a total of 104 sunflower fields were surveyed for sunflower downy mildew incidence and prevalence from June 30 to July 2 and July 7 to 10 of 2014 in North Dakota and northern South Dakota. Prevalence of downy mildew was 64.4% of the fields surveyed (67 of 104). Only 10 fields surveyed (9.6%) had incidence of 5% or greater and did not appear to be concentrated in any one geographic region (Figure 1.1). Of 104 fields surveyed, 26 fields (25.0%) were identified with percent incidence between 0.5 and 5% out of 200 plants assessed. An additional 31 fields (29.8%) were identified as having trace amounts of downy mildew where

downy mildew occurred in the field, but percent field incidence was 0%. The majority of the fields were surveyed while plants were in the vegetative growth stages, but some of them were early R1.

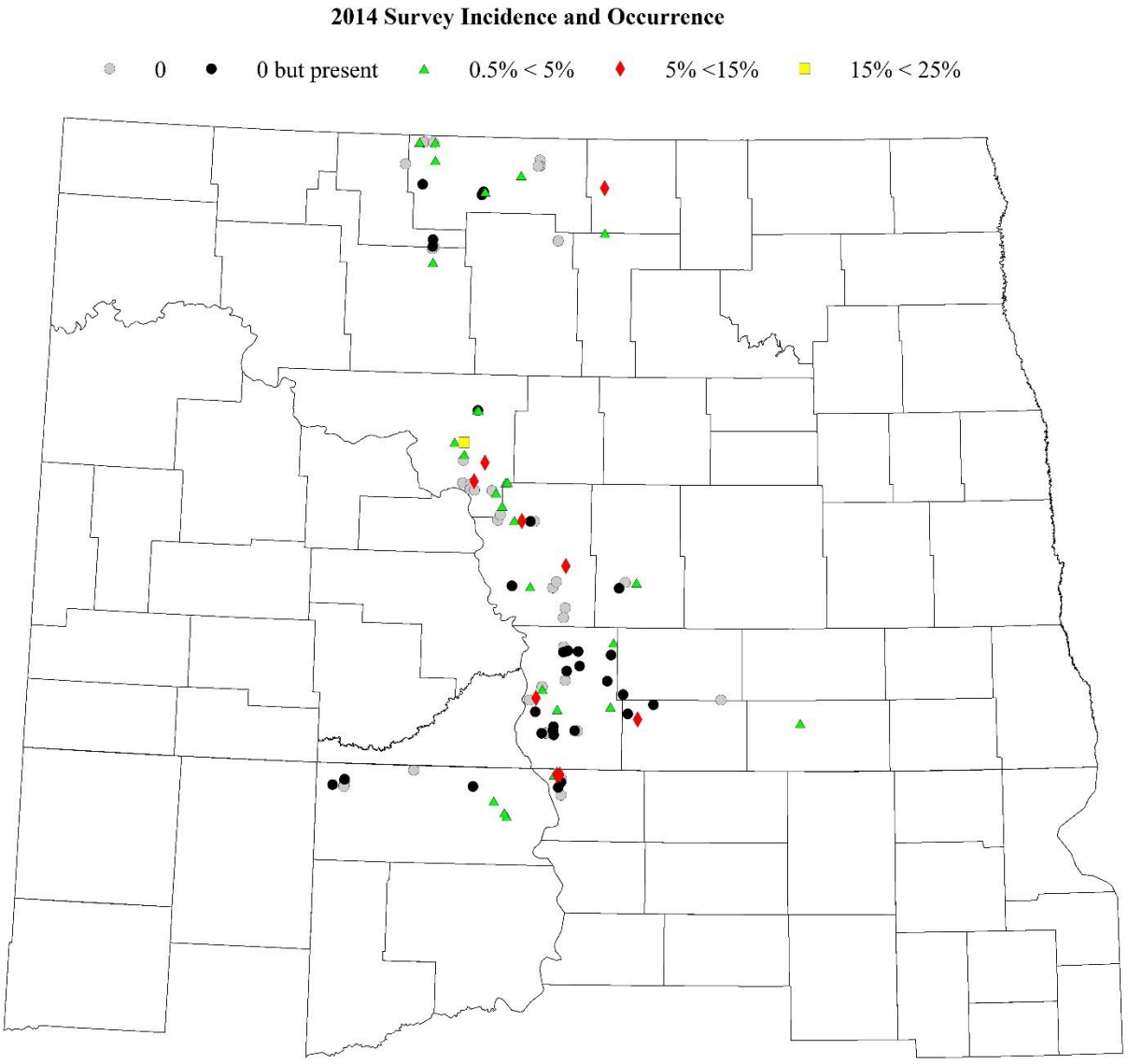

Figure 1.1. Locations and percent incidence of downy mildew in North Dakota and northern South Dakota in 2014. For this study, "0" indicates that no downy mildew was identified when calculating percent field incidence based on the 200 plants at survey points and "0 but present" indicates the occurrence of downy mildew in the field at trace amounts. Shapes indicate percent incidence by category.

In 2015, a total of 76 sunflower fields were surveyed from July 8 to 10 and from July 23 to 24 of 2015 in North Dakota and north central South Dakota. Prevalence of downy mildew was 77.6% of the fields (59 of 76). Sixteen fields surveyed (21.1%) had incidence of 5% or greater and were not concentrated in any one geographic region (Figure 1.2). In most fields, the infected plants seemed to be scattered throughout the field. Of 76 fields surveyed, 18 fields (23.7%) were identified with percent incidence between 0.5 and 5% out of 200 plants assessed. An additional 25 fields (32.9%) were identified as having trace amounts of downy mildew where downy mildew occurred in the field, but percent field incidence was 0%. Secondary downy mildew infection was observed in 22 of the 59 fields (37.3%) with downy mildew. Sunflowers in the majority of the fields were at R1.

No downy mildew was observed in 35.6% of fields in 2014 and in 22.4% of fields in 2015 (Figure 1.3). The percent of fields identified as having trace amounts of downy mildew where downy mildew occurred in the field, but percent field incidence was 0% was lower in 2014 than 2015, at 29.8% and 32.9%, respectively. The percent of fields with incidence between 0.5 and 5% was only slightly higher in 2014 than 2015, at 25% and 23.7%, respectively. Incidence was higher in 2015 than 2014 for the other three incidence categories. Percent of fields with incidence between 5 and 15% increased from 8.7% to 14.5%, percent of fields with incidence between 15 and 25% increased from 1.0% to 5.3% and one field was found in 2015 with percent incidence between 25 and 50%. Prevalence based on downy mildew observed anywhere in the field increased from 64.4% in 2014 to 77.6% in 2015.

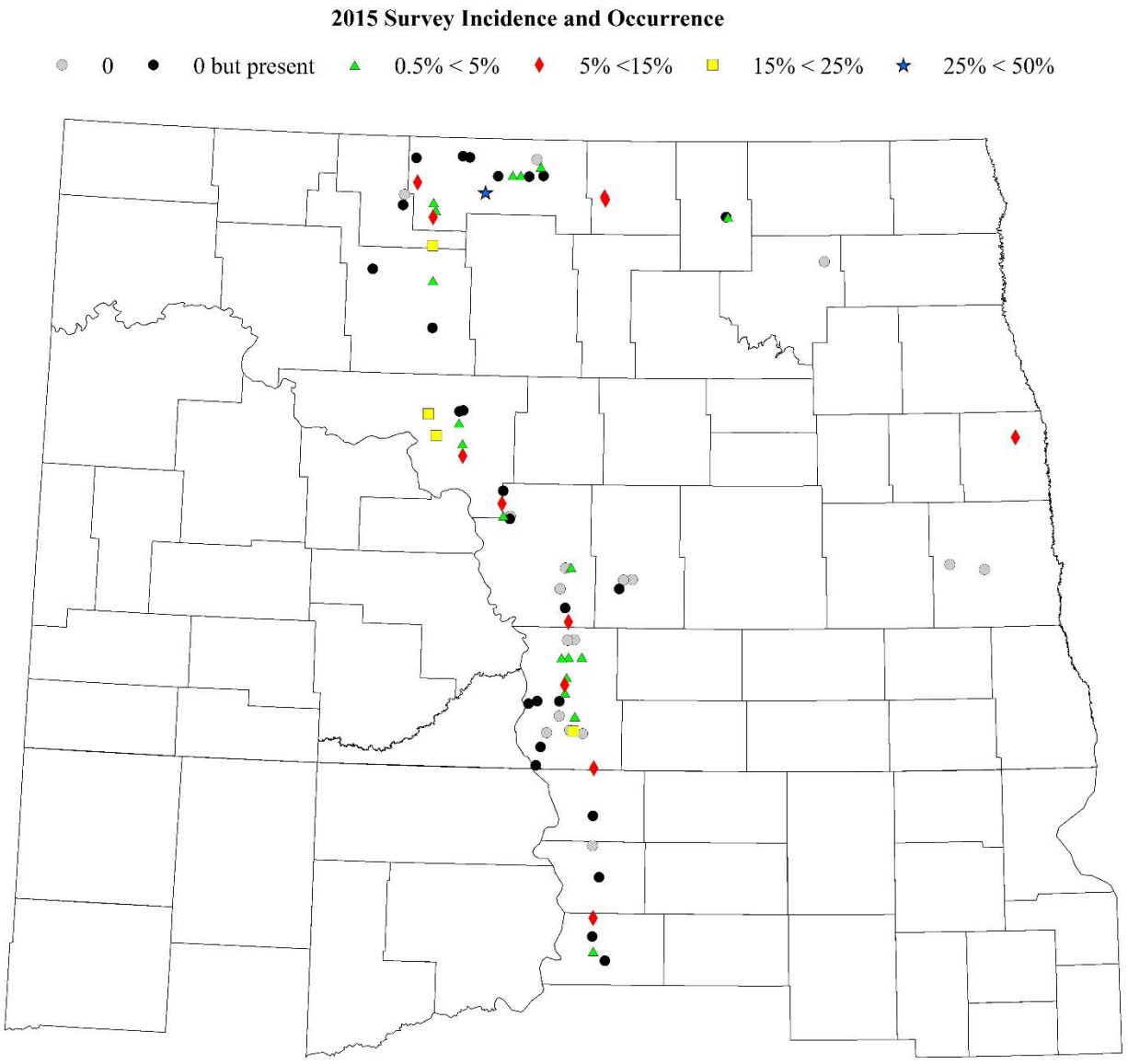

Figure 1.2. Locations and percent incidence of sunflower downy mildew in North Dakota and northern South Dakota in 2015. For this study, "0" indicates that no downy mildew was identified when calculating percent field incidence based on the 200 plants at survey points and "0 but present" indicates the occurrence of downy mildew in the field at trace amounts. Shapes indicate percent incidence by category.

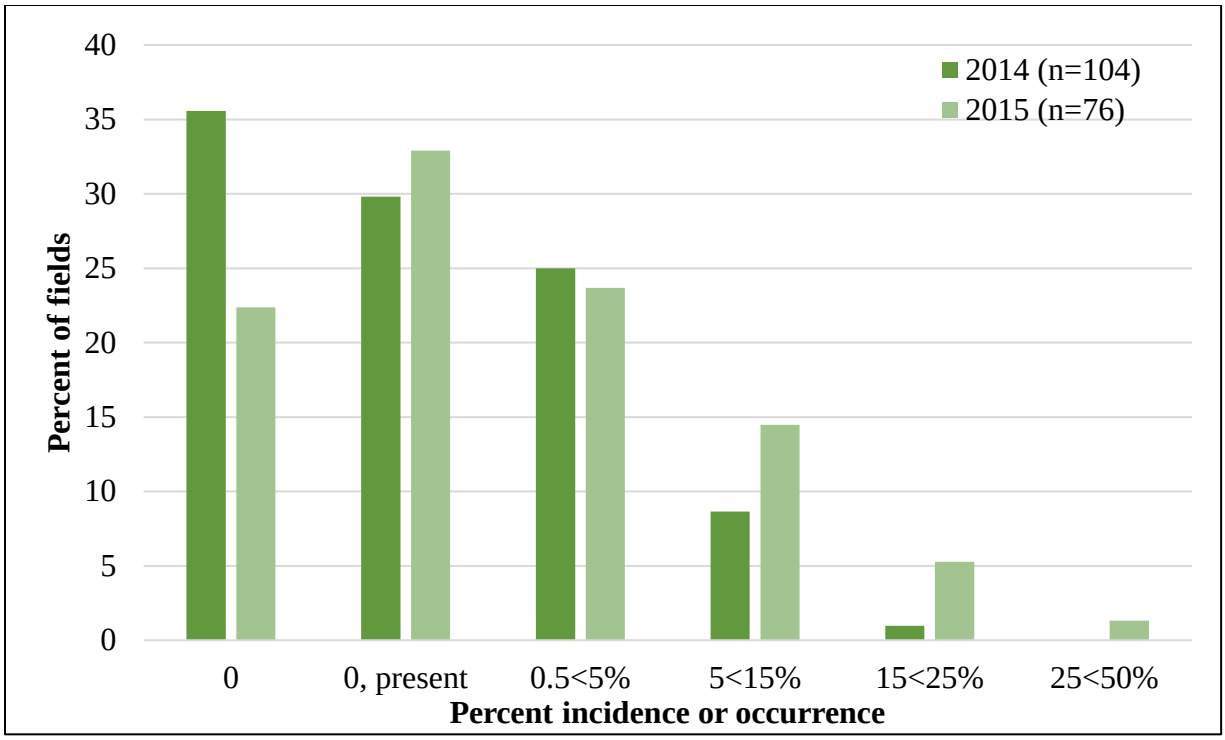

Figure 1.3. Percent incidence of downy mildew in North Dakota and northern South Dakota in 2014 and 2015. Percent of fields was calculated based on the number of fields in each category divided by the total number of fields. For this study, "0" indicates that no downy mildew was identified when calculating percent field incidence based on the 200 plants at survey points and "0 but present" indicates the occurrence of downy mildew in the field at trace amounts.

To more completely understand distribution of downy mildew in North Dakota, incidence and prevalence data that was generated by other methods was obtained from 2014 and 2015. Specifically, the NDSU-IPM survey, in which sunflower fields are scouted as part of a season-long monitoring program conducted in approximately five crops and the NSA survey, in which two locations in a field are scouted for yield limiting factors at the end of the season. For direct comparison of survey results in this project, only data from counties scouted in this work was used (Figures 1.4 and 1.5). Additionally, because free-soil moisture is so critical for disease development, rainfall data between May 1 and June 30, collected by North Dakota Agriculture Weather Network (NDAWN, http://ndawn.ndsu.nodak.edu/weather-data-daily.html, 2017) weather stations located in major sunflower growing areas of North Dakota (Bottineau, Turtle

Lake and Linton) was obtained for 2014 and 2015 (Figures 1.6 and 1.7). Total precipitation in May and June at Bottineau and Turtle Lake was similar in 2014 at 224 mm and 213 mm, respectively, and much lower in 2015 at 142 mm and 132 mm, respectively. Total precipitation for May and June at Linton in 2014 was 155 mm and in 2015 was 277 mm. Planting dates were estimated based on plant growth stage when fields were surveyed using the North Dakota Agricultural Weather Network (NDAWN, http://ndawn.ndsu.nodak.edu/ sunflower-growing-degree-days.html, 2017) application for sunflower growing degree days (GDD) and growth stages to calculate rainfall from just before planting to seedling emergence.

In 2014, fields 26 and 31 were located within 16 km of the Turtle Lake NDAWN station and had incidence levels of 16% and 12%, respectively. Field 29, 0% incidence but some diseased plants in the field, and fields 25, 27, 28 and 30 with 0.5% incidence were also in the vicinity. These fields were all surveyed while in vegetative plant stages between V8 and V12. If estimated planting dates were correct, then 26.4 mm of rain fell at the NDAWN station just after fields 25, 26 and 27 were planted May 28-30, 2014 and immediately before fields 29, 30 and 31 were planted. For field 28, 26.7 mm of rain fell at emergence on June 18, 2014.

In 2015, three additional groups of fields were near NDAWN stations. The first two fields were field 12 with 36% incidence and field 13 with 0% incidence, which were 15 and 5.6 km from the Bottineau NDAWN station. From June 2-6, 2015, within four to eight days after the estimated planting of field 12, 36.6 mm of rain fell at the NDAWN station. Field 13 was planted much earlier, and the seeds did not emerge for three weeks. Five days after the estimated planting date 15.5 mm of rain fell on May 6, 2015 at the NDAWN station and fifteen days after planting 40.9 mm of rain fell on May 16, 2015. The next group of seven fields was west of the Turtle Lake NDAWN station. Fields 28, 29, 33 and 34 had downy mildew disease incidence of

22.5, 16.5, 2.5 and 13%, respectively. The plants in these fields were at R1 and had 85 to 100% secondary downy mildew. The closest NDAWN station is 29.8, 25.7, 11.6 and 15.6 km away from these fields; however, it recorded precipitation of 26.2 mm in the four days before planting between May 14-17, 2015 and 22.0 mm at emergence, June 1-2, 2015. The other three fields in this area were all planted later with low levels of disease. They were in an arc 11.3 to 16.1 km from the Turtle Lake NDAWN station and north and east of the fields with high incidence. Fields 31 and 32 had 22.8 mm of rain recorded the day after planting, June 6, 2015, and field 30 had light sporadic rain recorded prior to emergence, between June 21 and 25, 2015. The last group of fields evaluated was near the Linton NDAWN station. Field 40 was the closest at 14.5 km away and fields 41, 53 and 54 were all about 24 km away. Fields 40 and 54 with 4.5 and 15.5% incidence, respectively, had 83.6 mm of rain recorded at the NDAWN station during the nine days prior to emergence between June 16 and 23, 2015, while fields 41 and 53 which were planted much earlier had 14.5 mm recorded in three events during the nine days prior to emergence between June 2 and 10, 2015 and no incidence of disease.

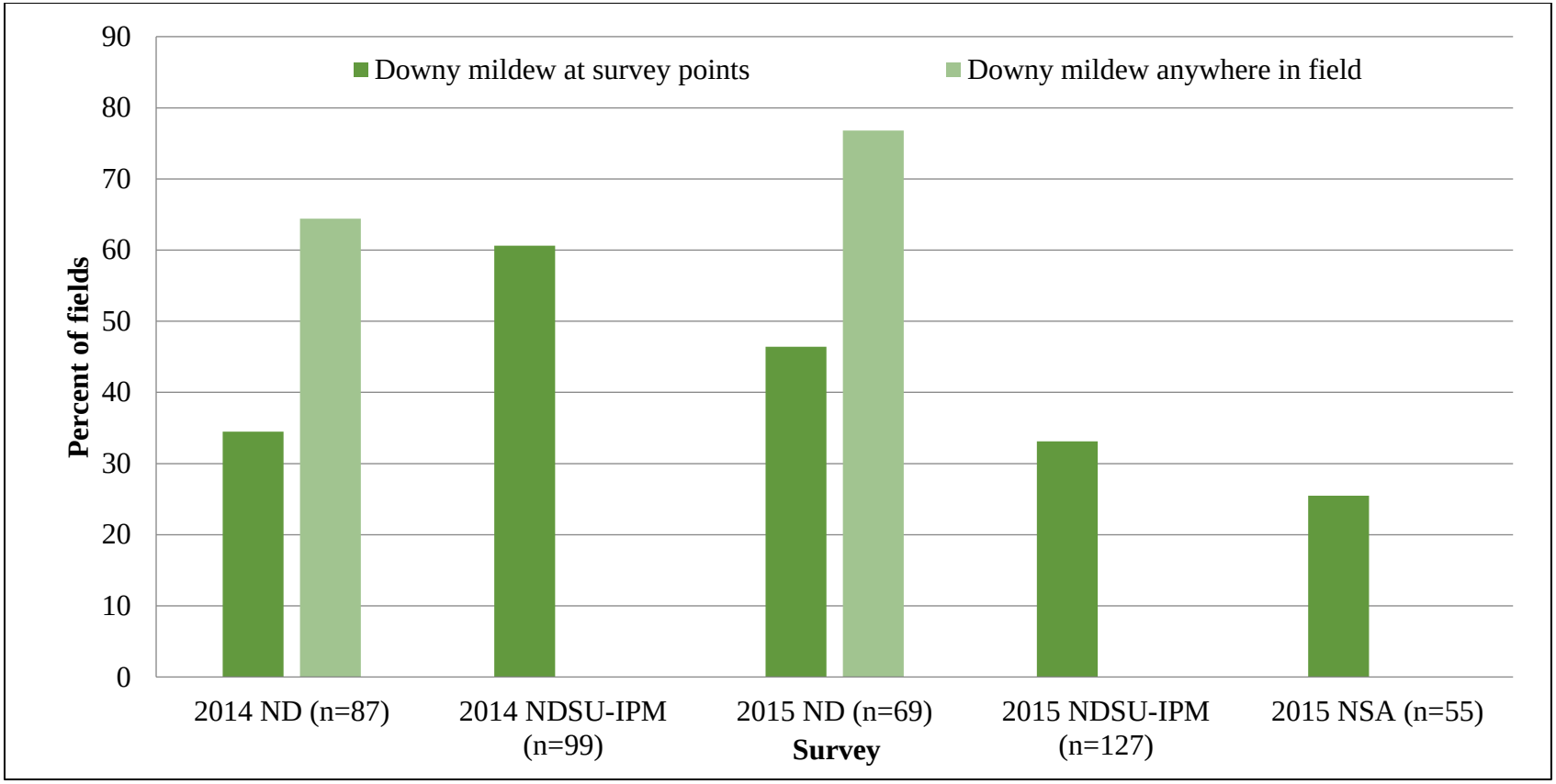

Figure 1.4. Prevalence of downy mildew in North Dakota fields in 2014 and 2015, as determined by the North Dakota State University-Integrated Pest Management (NDSU-IPM) survey (Knodel 2014; 2015), the National Sunflower Association (NSA) Crop Survey (Kandel and Gulya 2016) and this study. Only survey data acquired from the same counties as this study was considered. Percent of fields was calculated based on the number of fields where downy mildew was observed divided by the total number of fields in the survey. For this study, prevalence values were given for the two definitions of prevalence, downy mildew identified at survey points or downy mildew identified anywhere in the field.

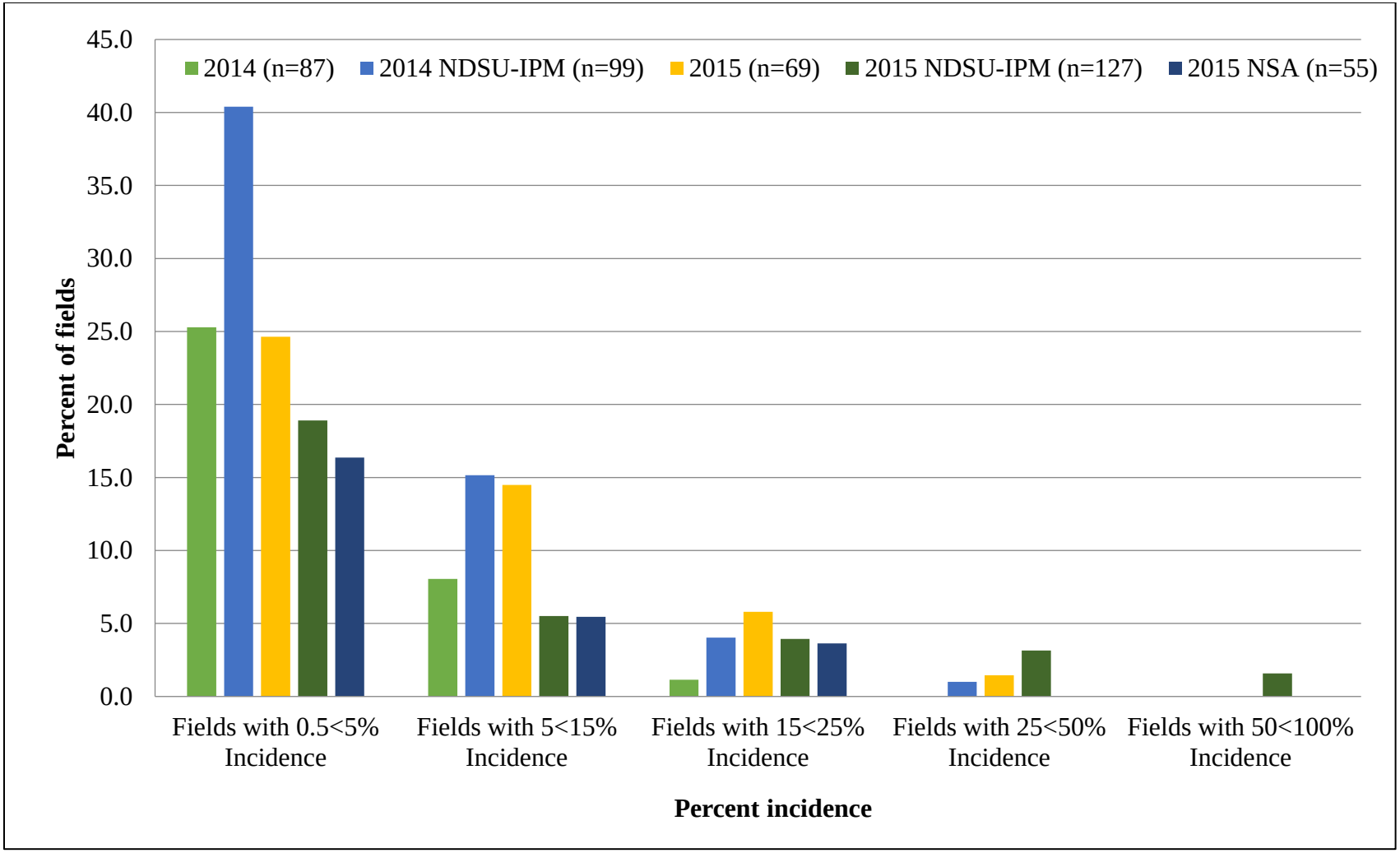

Figure 1.5. Percent of fields by incidence category in North Dakota in 2014 and 2015, as determined by the North Dakota State University-Integrated Pest Management (NDSU-IPM) survey (Knodel 2014; 2015), the National Sunflower Association (NSA) Crop Survey (Kandel and Gulya 2016) and this study. Only survey data acquired from the same counties as this study was considered. Percent of fields was calculated based on the number of fields where downy mildew was observed divided by the total number of fields in the survey**.**

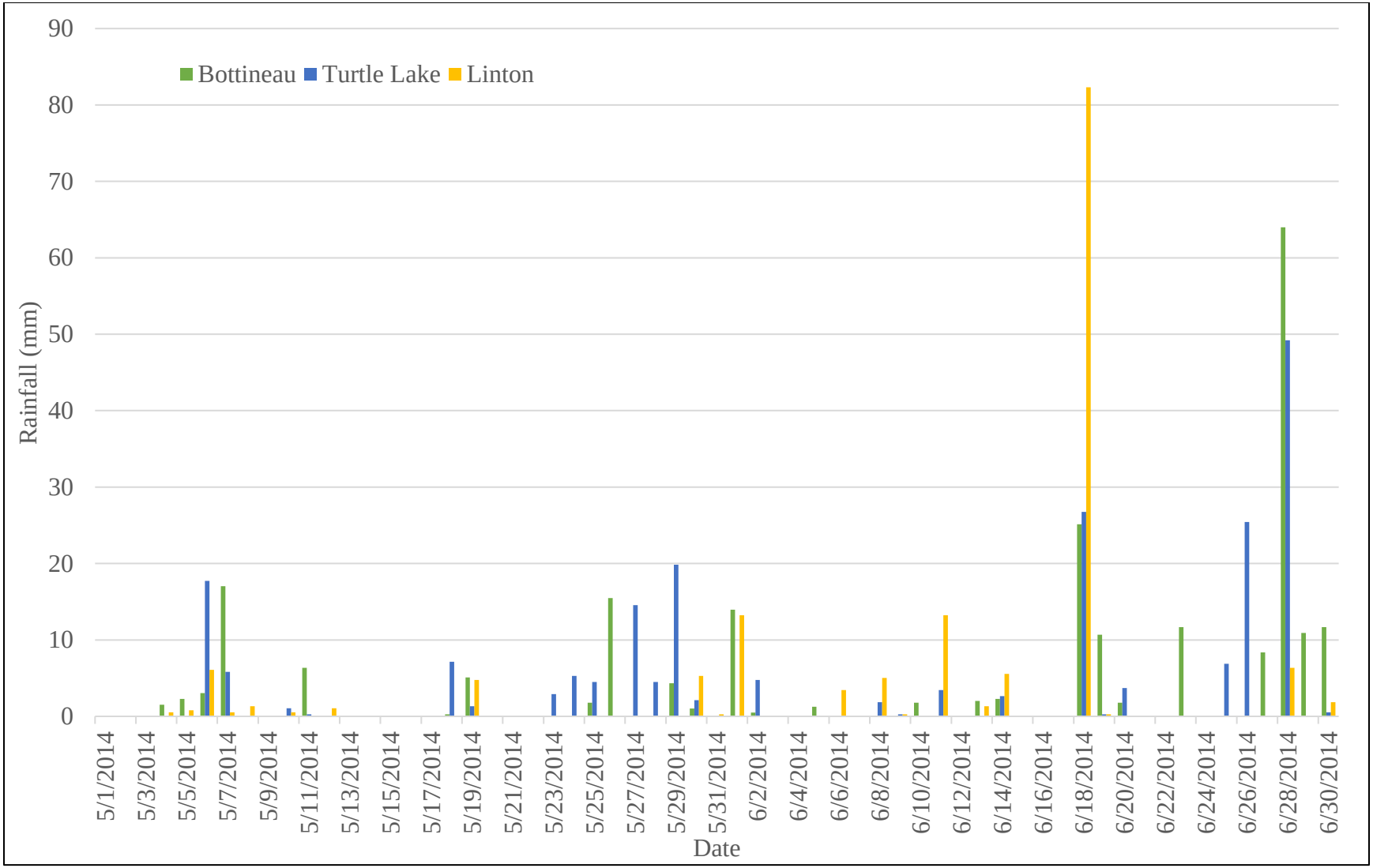

Figure 1.6. Rainfall data between May 1 and June 30, 2014, collected by North Dakota Agriculture Weather Network (NDAWN, http://ndawn.ndsu.nodak.edu/weather-data-daily.html) weather stations located in major sunflower growing areas of North Dakota (Bottineau, Turtle Lake and Linton).

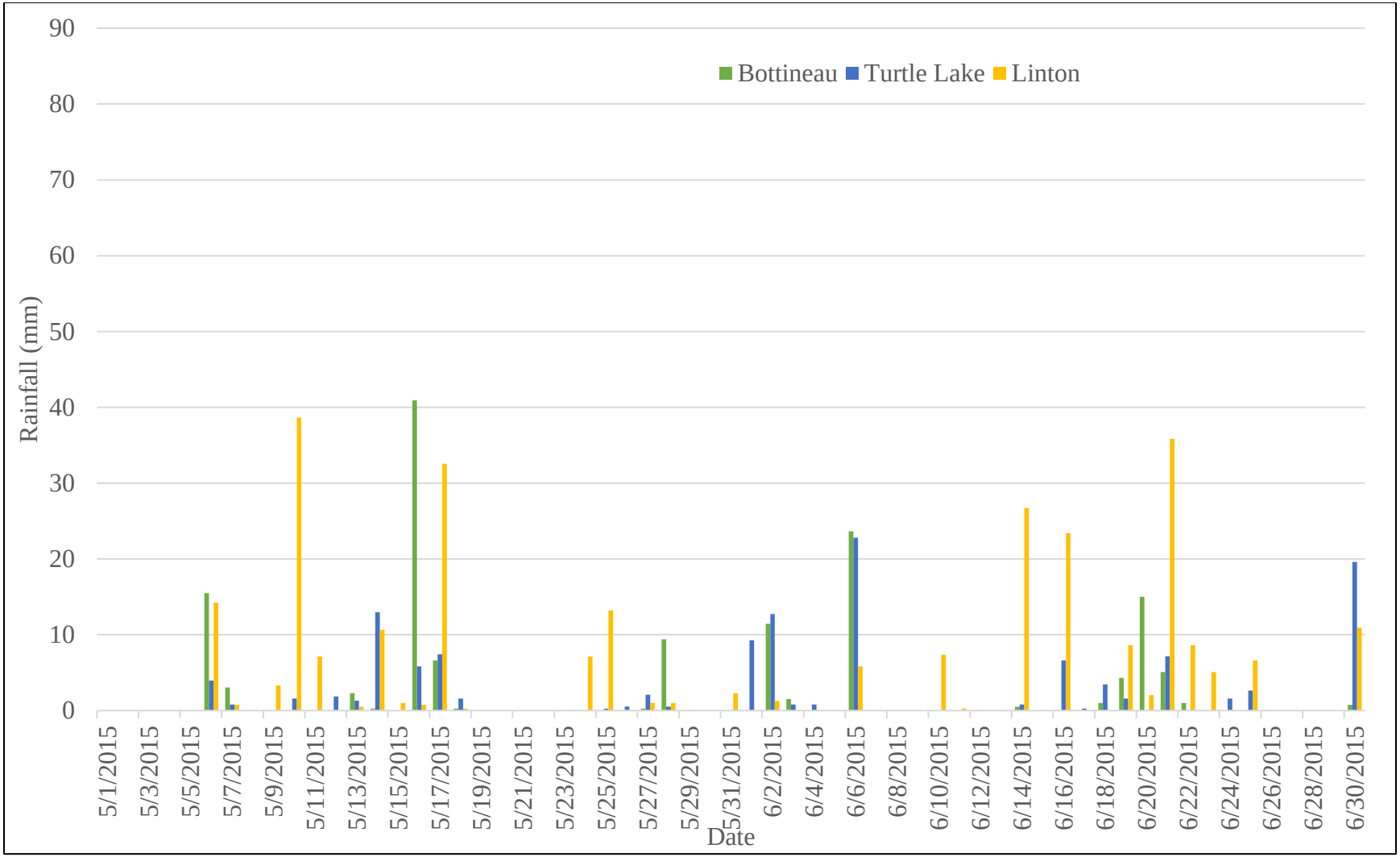

Figure 1.7. Rainfall data between May 1 and June 30, 2015, collected by North Dakota Agriculture Weather Network (NDAWN, http://ndawn.ndsu.nodak.edu/weather-data-daily.html) weather stations located in major sunflower growing areas of North Dakota (Bottineau, Turtle Lake and Linton).

Discussion

The objective of this *P. halstedii* disease survey conducted in 2014 and 2015 was to assess the distribution, incidence and prevalence of downy mildew in North Dakota and northern South Dakota in a survey designed specifically to evaluate downy mildew in the geographic areas with the most sunflower fields. As a consequence, timing of the survey and data points collected (prevalence and incidence) were specifically designed to detect and enumerate the disease. In both years, the majority of fields in the region had at least some downy mildew, but incidence for the vast majority of those fields was determined to be low.

Some aspects of the methodology and data collection in this survey differ from previous surveys, importantly; the objective of the survey, the time of year it was conducted, the way the disease was scouted and the geographic range of the surveys. Protocols to estimate field incidence were similar for this survey and the NDSU-IPM survey with a visual inspection for downy mildew signs and symptoms on 40 plants at five points in an inverted W-shaped pattern for a total of 200 plants. Field incidence for the NSA survey was based on 25 consecutive plants in two rows at two points in the field for a total of 100 plants. Both the NDSU-IPM and the NSA surveys only consider downy mildew diseased plants found at the survey locations. However, downy mildew is most often found clustered in wetter parts of fields where the soil does not drain well. The presence of downy mildew could easily go undetected if the selected survey points were not in an area of the field where downy mildew was present. It also is not scientifically sound to target one of these areas for a survey point. Therefore, this survey also includes prevalence which is based on the presence or absence of any plants with downy mildew found in the field. When plants with downy mildew found anywhere in the field were included when determining prevalence, values increased by approximately 30%. Prevalence for the

NSDU-IPM and NSA surveys is based only on field incidence, which is appropriate for these surveys since they are rating several diseases. The NSA survey rates and ranks anything that would limit yields including diseases, birds, missing plants, weeds and insect damage. Both the NDSU-IPM and the NSA surveys cover more counties in North Dakota than this survey.

To more directly compare data generated from this survey to data collected in the two other surveys, survey data was included only for the same counties in North Dakota. For North Dakota, prevalence values were higher in 2015 than 2014 for this survey, while the reverse was true for the NDSU-IPM survey. Prevalence based on incidence at the survey locations was 34.5% in 2014 and 46.4% in 2015. Prevalence based on downy mildew anywhere in the field was 64.8% in 2014 and 76.8% in 2015. For the NDSU-IPM survey based on the same counties, prevalence was 60.6% in 2014 and 33.1% in 2015. The mean disease incidence for this survey in 2014 (1.0%) was much lower than the mean incidence for the NDSU-IPM survey (3.1%) and the percent of fields in each incidence category was lower for this survey as well. One possibility in the difference between surveys is that this survey was conducted too early in 2014 and more infected plants would have been visible later. In 2015, mean incidence values were similar for both this survey, 3.3%, and the NDSU-IPM survey, 2.9%. However in 2015, incidence was higher for this survey for the lower three incidence categories between 0.5 and 25% incidence and incidence was higher in the NDSU-IPM survey for incidence greater than 25%. Therefore, in the NDSU-IPM survey for 2015, the zero incidence in 66.9% of the fields compensated for two fields with very high incidence values, which indicates that arbitrary selection of fields causes a portion of the variability between the surveys.

When compared to this survey and the NDSU-IPM survey, the late-season NSA survey in 2015 appears to have underestimated the prevalence of downy mildew in a field when data was

limited to the same counties in North Dakota. Prevalence in 2015 was 25% for the NSA survey, 33% for the NDSU-IPM survey, 46% for this survey and 77% for this survey based on the presence of diseased plants in a field. Mean incidence was also much lower for 2015 for the NSA survey. A portion of these differences was likely due to the early season death of downy mildew plants. Another possible reason for the differences could be variability due to the fact that only half the number of plants are evaluated in the NSA survey.

In an attempt to compare early-season with late-season disease incidence, 14 fields were evaluated in both this survey in 2015 and the 2015 NSA survey. Seven fields with zero incidence remained the same between this survey and the NSA Survey. Disease incidence in three fields with low incidence in this early survey (0.5%) remained low in the late-season survey with incidence levels of 1, 2 and 5%, while in one field disease incidence increased from 6 to 20%. In two fields, incidence decreased between surveys from 13 to 0% and 16.5 to 4%. These decreases could have been due to plants that died early and decayed or sampling locations. In the final field with the highest incidence, incidence values remained similar at 22.5% and 22%.

Downy mildew signs and symptoms were easier to identify when sunflower plants were in late vegetative stages or early in the reproductive stages (R1). The timing of this period was earlier in 2015 than 2014 and the survey was started earlier in 2014 as well. This timing may have decreased field incidence values for 2014 for this study. The timing of the 2015 survey was ideal, as the majority of plants were at R1. Scouting of most of the fields for the NDSU-IPM surveys was conducted between late vegetative stages and reproductive stage R3 and the NDSU-IPM survey seems to be an excellent tool for monitoring prevalence and incidence of downy mildew in North Dakota. Because of the sporadic nature of downy mildew in a field, a notation

about downy mildew being found in trace amounts in a field outside the survey points provided additional useful information and increased the prevalence of downy mildew by about 30% both years of the survey. The prevalence and incidence of downy mildew from year to year has also been quite variable, so conducting the NDSU-IPM survey each year is advantageous.

To specifically assess the variable impact of precipitation, a comparison using individual fields from 2014 and 2015 with high disease incidence to nearby fields with low disease incidence was made. Statewide rainfall totals between May 15 and June 15 were not significantly correlated to downy mildew disease incidence between 2001 and 2011 (Gulya et al. 2013). Planting dates were estimated based on plant growth stage when fields were surveyed using the North Dakota Agricultural Weather Network (NDAWN, http://ndawn.ndsu.nodak.edu/sunflower-growing-degree-days.html, 2017) application for sunflower growing degree days (GDD) and growth stages and rainfall from just before planting to seedling emergence was acquired from the closest NDAWN station. In 2014, fields 26 and 31 were located within 16 km of the Turtle Lake NDAWN station and had incidence levels of 16% and 12%, respectively. Field 29, with 0% incidence but some diseased plants in the field, and fields 25, 27, 28 and 30 with 0.5% incidence were also in the vicinity. Greater than 26 mm of rain fell at the NDAWN station in the vicinity of these seven fields just after three fields were planted, immediately before three fields were planted and at emergence for one field. Incidence of downy mildew could have become significant in all of these fields, yet it only became significant in one of the fields planted right after rain and one of the fields planted right before rain.

In 2015, three additional groups of fields were evaluated for precipitation effects. The first two fields were field 12 with 36% incidence and field 13 with 0% incidence near the Bottineau NDAWN station. Within four to eight days after the estimated planting of field 12,

36.6 mm of rain fell at the NDAWN station. Field 13, which was planted much earlier received 15.5 mm of rain five days after the estimated planting date and 40.9 mm of rain fifteen days after planting. The next group of seven fields was west of the Turtle Lake NDAWN station. Fields 28, 29, 33 and 34 had downy mildew disease incidence of 22.5, 16.5, 2.5 and 13%, respectively. Unfortunately, the closest NDAWN station was between 11.6 and 29.8 km away from these fields; however, it recorded precipitation of 26.2 mm in the four days before planting and 22.0 mm at emergence. The other three fields in this area were all planted later with low levels of disease. They were in an arc 11.3 to 16.1 km from the Turtle Lake NDAWN station and north and east of the fields with high incidence. Fields 31 and 32 had 22.8 mm of rain recorded the day after planting and field 30 had light sporadic rain recorded prior to emergence. The last group of fields evaluated was near the Linton NDAWN station. Fields 40 and 54 with 4.5 and 15.5% incidence, respectively, had 83.6 mm of rain recorded at the NDAWN station during the nine days prior to emergence, while fields 41 and 53 had 14.5 mm recorded during the nine days prior to emergence and no incidence of disease. In 2015, eight fields with higher disease incidence are associated with rain, two fields with little disease incidence are associated with rain and three fields with low incidence are believed to have had little rainfall prior to emergence. The major variables that could all significantly affect disease incidence and that were not known were the type of sunflowers planted, the genetic resistance and whether the seed was treated with a fungicide. The difficulty in correlating rainfall to disease incidence was very clear after evaluating these four groups of fields from 2014 and 2015. It does take soil moisture to cause disease, but disease will not necessarily develop just because it rained.

The secondary purpose of this survey was to collect downy mildew isolates to establish baseline sensitivity to azoxystrobin and monitor for the development of fungicide resistance and

P. halstedii virulence phenotype changes and this evaluation would have been much less useful without field incidence data for comparison. Additionally, this survey was able to show that while downy mildew was present in many fields, incidence was typically low. Rainfall of greater than 14 mm of rain occurred after planting and prior to emergence in all the fields evaluated with higher incidence, but current management practices including sunflower type, resistance genes and fungicides appear to be limiting disease incidence when it rains prior to seedling emergence.

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CHAPTER 2. DETERMINATION OF VIRULENCE PHENOTYPES OF *PLASMOPARA HALSTEDII* IN THE NORTH CENTRAL UNITED STATES

Introduction

Downy mildew, caused by the biotrophic oomycete pathogen *Plasmopara halstedii* (Farl.) Berl. and de Toni, is an economically important disease in cultivated sunflowers, *Helianthus annuus* L. Both sunflowers and *P. halstedii* originated in North America (Kolte 1985; Leppik 1966; Novotelnova 1966). Cultivated sunflower is grown primarily as an oilseed crop and is produced on every continent except Antarctica. *Plasmopara halstedii* is assumed to have spread internationally primarily through contaminated seed, and causes yield loss in every region sunflower is produced except Australia and New Zealand (CABI 2016; Constantinescu and Thines 2010; Kolte 1985; Leppik 1966).

Qualitative genetic resistance, conferred by single dominant resistance genes denoted *Pl* (for *Plasmopara*), is one of the most important tools for sunflower downy mildew management (Tourvieille de Labrouhe et al. 2008). Single gene resistance can be overcome quickly, if the same single resistance is used consecutively (Tourvieille de Labrouhe et al. 2010). Pyramiding single resistance genes or rotating hybrids with different genes in the field would make resistance more durable (Tourvieille de Labrouhe et al. 2008; 2010). However, seed companies do not label their seed with the resistance gene used, making gene rotation by growers nearly impossible. Pyramiding multiple resistance genes is difficult due to the time and resources required using classical breeding techniques and the limited molecular markers available. Many previously deployed resistance genes have been overcome by the pathogen (Tourvieille de Labrouhe et al. 2008). Consequently, periodic monitoring of pathogen changes remains important for breeding and selecting resistant hybrids.

Beginning in the 1960's, genetic resistance identified in cultivated oilseed sunflowers was introgressed into inbred lines (Vear et al. 2008a). Maintainer line HA60 was released in 1968 and restorer lines RHA 265 and RHA 266 were released in 1971 with Canadian line CM953-102 containing resistance gene *Pl*$_1$ as part of their pedigree (Sunflower inbreds 2006b; Vear et al. 2008a). Restorer line germplasm RHA 271, RHA 273 and RHA 274 were released in 1973, all with resistance gene *Pl*$_2$ from HA 62, a sister selection to maintainer line HA 61, released in 1968 (Fick and Zimmer 1974). The pedigree for HA 61 and HA 62 includes Canadian line CM953-88 which is believed to provide the downy mildew resistance (Sunflower inbreds 2006a; Vear et al. 2008a). While RHA 274 also contained the resistance gene *Pl*$_1$, four other genes (*Pl*$_9$, *Pl*$_{10}$, *Pl*$_{11}$ and *Pl*$_{12}$) were also identified in the line later (Gulya et al. 1991b; Liu et al. 2012; Molinero-Ruiz et al. 2003; Rahim et al. 2002). These first resistance genes deployed were effective in North America until the pathogen developed virulence to all of them by 1980 (Carson 1981; Miller and Gulya 1984).

In the 1980's, an increased effort to identify downy mildew resistance from wild *Helianthus* germplasm began. DM-2, a composite of plants originating from the open-pollinated accession Novinka with resistance from *H. tuberosus*, was released in 1984 with resistance genes *Pl*$_5$, *Pl*$_{11}$ and *Pl*$_{12}$ (Liu et al. 2012; Miller and Gulya 1984; Rahim et al. 2002; Vear et al. 2008a). HA-R4 and HA-R5 were also released in 1984 and contained resistance genes *Pl*$_{16}$ and *Pl*$_{13}$, respectively, both derived from cultivated sunflowers (Liu et al. 2012; Mulpuri et al. 2009; Vear et al. 2008a). Downy mildew resistance in HA-R4 is believed to be derived from an Argentinean pool with Russian open-pollinated cultivars crossed with *H. annuus*, *H. argophyllus* and *H. petiolaris* in 1955 and 1956 (González et al. 2015; Vear et al. 2008a). Downy mildew resistance in HA-R5 is believed to come in part from Canadian line 953-102 (González et al. 2015; Vear et

al. 2008a). In 1986, the USDA released six downy mildew resistant lines: *Pl*$_6$ in HA 335 and HA 336 from wild *H. annuus*, *Pl*$_7$ in HA 337, HA 338 and HA 339 from *H. praecox* and *Pl*$_8$ in RHA 340 from *H. argophyllus* (Miller and Gulya 1991).

During the 1990's, some of the new downy mildew resistant lines, including those containing resistance genes *Pl*$_5$, *Pl*$_6$, *Pl*$_7$ and *Pl*$_8$, were used by seed companies to develop commercial hybrids (Vear et al. 2008b). Two downy mildew resistant lines were released in 1999 by USDA from *H. argophyllus*, RHA 419 and RHA 420 (Miller et al. 2002). The gene in these two hybrids was called *Pl*$_{Arg}$, breaking from the traditional numbering of resistance genes even though it was not the first resistance gene found from *H. argophyllus* (DuBle et al. 2004; Imerovski et al. 2014; Vear et al. 2008a).

Several additional lines with new downy mildew resistance genes have been released since 2000, including HA 458 released in 2006 with gene *Pl*$_{17}$ from wild *H. annuus*, HA DM 1 released in 2015 with gene *Pl*$_{18}$ from *H. argophyllus*, and RNID, a proprietary inbred line from Nidera, S.A. in Argentina, with resistance gene *Pl*$_{15}$ (Bertero de Romamo et al. 2010; DuBle et al. 2004; Paniego et al. 2012; Qi et al. 2015; 2016; Vear et al. 2008a). In 2001, RHA 436, RHA 437 and RHA 438 were released with *Pl*$_8$ from RHA 340 and high oleic germplasm (Miller et al. 2004). In 2006, RHA 468 was released with *Pl*$_{Arg}$ from RHA 419 (B. Hulke, *personal communication*). In 2008, HA 460, a maintainer line, was released with *Pl*$_8$ from RHA 340 and high oleic germplasm (Hulke et al. 2010). *Pl*$_{19}$ from wild *H. annuus* has been introgressed into confection sunflower and it is hoped that *Pl*$_{19}$ will be combined with *Pl*$_{Arg}$ and *Pl*$_{18}$ into a single line for durable downy mildew control in confection sunflowers (Zhang et al. 2016).

Plasmopara halstedii has quickly been able to adapt to, and overcome, many resistance genes deployed. This is due at least in part to the highly variable nature of the pathogen, but also

to the large number of major genes that have been deployed singly in a global production system (Markell et al. 2016a; Virányi et al. 2015). *Plasmopara halstedii* virulence to resistance genes Pl_1 and Pl_2 led to the development of the first two virulence phenotypes of *P. halstedii* (Sackston 1981). The first two virulence phenotypes were separated geographically, so the "European" race and the "North American" race (syn: "Red River" race) were easily distinguished (Gulya et al. 1997). As the number of resistance genes and virulence phenotypes increased, a nomenclature system was needed to distinguish virulence phenotypes. Initially, a simple numbering system was used in the United States, where the "European" race became known as "Race 1" and the "North American" race became known as "Race 2" (Gulya et al. 1997). "Race 3" was recognized in the United States in 1980 and by 1990, the United States named "Races 4 and 5" (Carson 1981; Sackston et al. 1990). Simultaneously, France had developed a different race nomenclature, where letters were used to denote different virulence combinations. As the number of "races" increased in both countries, the two different nomenclatures impeded communication and the deployment of effective resistance internationally (Gulya et al. 1998). In an attempt to address this, a 1990 proposal was made by Canada and the United States to develop nomenclature using a gene based system to compare virulence phenotypes between countries (Sackston et al. 1990). In 1991, the proposed gene based virulence formulas for the first eight races were published, with Race 8, for example, becoming known as Race 1,2,3,4,a,b (Gulya et al. 1991a). However, this was not adopted because it was deemed cumbersome and because some of the *Pl* genes are actually clusters of genes which provide resistance to one or more races (Gulya et al. 1998; Vear et al. 1997).

In 1998, an international proposal identified nine standardized, publicly available, fixed inbred lines with consistent downy mildew reactions and different resistance genes that could be

used as differential lines, and when used in three sets of three, an easy, concise race nomenclature system (Gulya et al. 1998). The following differential lines were proposed: HA 304, RHA 265, RHA 274, PMI3, PM 17, 803-1, HA-R4, QHP1 and HA 335 (Gulya et al. 1998). New differential lines were to be added as needed in sets of three (Gulya et al. 1998).

In 2000, after testing the existing races of downy mildew on the proposed standardized set of nine differential lines, ten races were described and the new nomenclature was presented at the International Sunflower Conference in Toulouse, France (Tourvieille de Labrouhe et al. 2000). After confirming the reactions of the lines, it was also proposed that "susceptible" meant sporulation had to occur on the true leaves (Tourvieille de Labrouhe et al. 2000). Only differential lines D-3 (RHA 274), D-8 (HA-R4) and D-9 (HA 335) have consistently been accepted by scientists (Trojanová et al. 2017). The differential line and the gene associated with it cannot be changed without destroying the continuity of races, aggregate virulence phenotypes, but because some of the sunflower lines selected can either be difficult to grow or difficult to rate for virulence they are regularly discussed and changed. In the United States, Dr. Gulya substituted a suitable hybrid for differential D-1, the susceptible check, and used DM-2 for differential D-4 instead of PMI3, an INRA selection of USDA composite DM-2, and HA-R5 for differential D-8 instead of QHP1, an INRA cross with HA-R5 (T. Gulya, *personal communication)*.

In 2012, Institut National de la Recherche Agronomique (INRA) proposed changes to two of the original nine differential lines; GB would replace HA 304, the susceptible check, for differential D-1 and QHP2 would replace QHP1 for differential D-8 (Tourvieille de Labrouhe et al. 2012). These two sunflower lines were not ones that had been utilized in the United States. Six differential lines, Y7Q, PSC8, XA, PSS2RM, VAQ and RHA 419, were proposed to

distinguish additional virulence not represented by genes in the original lines (Tourvieille de Labrouhe et al. 2012). These proposed differential lines have not been internationally endorsed and will be referred to as the INRA proposed differential lines (Trojanová et al. 2017).

The pathogen continues to evolve under selection pressure, enabling it to overcome resistance genes. Currently, 42 races of *P. halstedii* have been found internationally and 23 races have been found in the United States (Virányi et al. 2015). A single isolate virulent on all nine of the internationally accepted differential lines has not yet been found; however, virulence to all nine lines has been found (Gascuel et al. 2015; Virányi et al. 2015). Between 2009 and 2013 nine different races and 38% of 520 isolates overcame the Pl_6 gene in the United States (Gulya et al. 2014; Virányi et al. 2015). As of 2013, virulence on the Pl_8, Pl_{Arg}, Pl_{15} and Pl_{17} genes and an unknown *Pl* gene in TX 16R had not yet been found in the United States (Gulya et al. 2014; Virányi et al. 2015). Virulence on the Pl_{16} and Pl_{13} genes was found in 2.8% of 470 isolates collected in the United States between 1998 and 2009 and was rarely found between 2010 and 2013 (T. Gulya, *personal communication*). Periodic surveys should be undertaken to monitor development of new virulence phenotypes as well as virulence against new lines with different genetic resistance. The objectives of this project are to determine the races of *P. halstedii* in the North Central Great Plains using the Standard set of nine differential lines and to evaluate INRA proposed differential lines and additional lines containing newer resistance genes that may have been utilized in hybrids by seed companies for their utility in elucidating additional virulence phenotypes present in the pathogen population.

Materials and Methods

Pathogen Material and Increase from Field Collection

In 2014, 187 isolates were collected from 68 fields in North Dakota and northern South Dakota and an additional 37 isolates were collected and submitted by personnel from the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), state Extension services, and seed companies. In 2015, 159 isolates were collected from 59 fields in North Dakota and South Dakota and an additional 88 isolates were collected and submitted from North Dakota, South Dakota, Minnesota and Nebraska by personnel from USDA-ARS, state Extension services, and seed companies.

Collections were made by destructively collecting sunflower plants with signs and symptoms of infection. Selected plants were a minimum of 30 m apart. When possible, a minimum of three downy mildew infected plants per field were collected, but more were collected in a few instances when spores appeared old. Plants with zoosporangia were preferentially selected over plants that were only chlorotic. One whole plant with the roots removed or several leaves from one plant was considered to be an isolate. All bagged isolates for each field were labeled and placed in a larger reclosable plastic bag and placed above ice in coolers.

To increase *P. halstedii* field isolates, susceptible sunflower seedlings were inoculated in a zoosporangia suspension prepared from symptomatic leaves from one isolate using methods described by Gulya (1996) within a week of their collection. Occasionally an isolate would have chlorosis, but not have many zoosporangia; therefore, the leaves were placed in the mist chamber on 100 mm petri dishes on top of dampened machine cloth on trays for 24-48 hours in order to

induce fresh sporulation before inoculating susceptible seedlings. Susceptible seedlings were continuously prepared for inoculation throughout the survey.

Two susceptible sunflower hybrids, Mycogen 270 and Mycogen 8N358CL, were selected for pathogen increase because they germinate quickly and contain no downy mildew resistance genes. To sterilize seeds before germination, seeds were soaked in 8.25% sodium hypochlorite (Chlorox™), deionized water and dish detergent for up to 15 minutes before rinsing thoroughly. Seeds were spread in a single layer on moistened machine cloth on a tray and placed in a germination chamber until radicles were at least one cm long. The germination chamber was kept turned off to prevent water condensing in the top of the chamber and raining down on the seedlings. As soon as possible after the seedling radicles were one cm long, they were used or they were placed in crisper boxes with moistened blue blotter paper and refrigerated.

To increase pathogen isolates, two to six infected leaves per isolate (depending on the visual quantity and quality of spores) were placed in a sample bottle containing approximately 80 ml of a 10 mM calcium chloride solution. The calcium chloride solution increases the amount of infection and was made by adding 20 ml of a two molar calcium chloride solution to 3.6 l of deionized water (Gulya 1996). The bottle was shaken multiple times in a 15 to 20-minute period. This spore suspension and 20 to 35 pre-germinated seedlings were mixed in two-ounce disposable plastic cups and placed in a dry, dark mist chamber for 3-6 hours at 16-18°C. Once inoculation was completed, infected seedlings were transferred to a sand:perlite (3:2 in 2014 and 3:1 in 2015) substrate and placed in a greenhouse with a 16-hour photoperiod at 20-30ºC. The substrate was put in 26 cm square inserts (T.O. Plastics, Clearwater, MN) placed in 27 x 54 cm standard web flats (T.O. Plastics, Clearwater, MN). Inserts were filled and leveled to about ¾ full, were gently watered and then five trenches were made with the triangular tip of a bottle

opener. The inoculated seedlings were placed in the shallow trenches with the roots down using tweezers sterilized with 70% alcohol. Seedlings were covered with a thin layer of pure sand approximately 5 mm thick and then lightly watered. Seedlings were replanted if they were growing down instead of up or were laying on the surface. Seed coats were removed a few days after emergence to enable better sporulation. Seedlings were grown out in a greenhouse until plants were beginning to develop true leaves. This took from eight to eleven days depending on greenhouse temperatures.

To induce sporulation, plants were placed in a dark mist chamber at a temperature of 16-18°C for 16-48 hours. Flats were watered lightly (if at all) on the day the plants were ready for sporulation, which both increased sporulation and prevented the water in the flats from dripping onto other flats when they were moved. The walls and door of the mist chamber and the seedlings were sprayed with a fine mist of water to create 100% relative humidity. After sporulation, each isolate in its square insert was separated from other isolates for several hours until dry. The cotyledons covered with zoosporangia were clipped with sterilized scissors, desiccated, and stored in 1.8 ml cryotubes at -80ºC. Cotyledons were desiccated with 8 mesh Drierite desiccant (calcium sulfate) for three to five days in 2014 and five to ten days in 2015 at room temperature. Approximately four cryotubes with cotyledons from seven to nine plants were stored for each isolate. Seedlings for subsequent increases were stored in small plastic bags in the refrigerator for up to one week.

Pathogen Increase from Storage

To produce fresh pathogen zoosporangia, 1.8 ml cryotubes containing sporulated, desiccated cotyledons were removed from storage in a -80ºC freezer. The selected cryotubes were heat shocked in warm water around 38°C for one to two minutes. Infected cotyledons were

placed in labeled two-ounce disposable plastic cups with about 30 pre-germinated seedlings of the susceptible hybrid, Mycogen 270, in 35 ml of a 10 mM calcium chloride solution for at least five hours. The sporulated cotyledons were placed with the inoculated seedlings in the 26-cm inserts in the sand:perlite blend before the inoculated seedlings were covered with a light layer of sand. After 10 to 11 days in the greenhouse, the plants were transferred to the mist chamber for sporulation. Then, eight to twelve seedlings were selected for virulence phenotyping and used immediately or left to dry for a few hours and then stored in plastic bags in the refrigerator. The remainder of the sporulated cotyledons for each isolate was clipped, desiccated and stored at -80°C. If only a few susceptible seedlings were visibly infected, the seedlings were used to inoculate another 35 susceptible seedlings.

Determination of Virulence Phenotypes

To determine virulence phenotypes, the standard United States set of nine differential lines, Mycogen 270, RHA 265, RHA 274, DM-2, PM 17, 803-1, HA-R4, HA-R5 and HA 335, and the six additional INRA proposed differential lines, Y7Q, PSC8, XA, PSS2RM, VAQ and RHA 419, were used (Table 2.1). For this project, the susceptible hybrid, Mycogen 270, and the other two substituted sunflower lines, DM-2 and HA-R5, rather than the internationally endorsed sunflower lines according to Tourvieille de Labrouhe et al. (2000), were used and are referred to as the Standard United States set of nine differential lines or the Standard set of nine differential lines. In addition, isolates were screened using the following additional USDA released lines containing resistance genes that may have been utilized in hybrids by seed companies: RNID, RHA 340, HA 458, HA DM 1, RHA 468, TX 16R and RHA 428.

Table 2.1. Sunflower differential lines and supplemental lines used to determine virulence phenotype of *Plasmopara halstedii* isolates in this study.

Triplet Code		U.S. Standard Differential Lines[a]		Resistance Gene[d]	Origin[e]
Digit	Value				
	1	D-1	Mycogen 270	None	
1st	2	D-2	RHA 265	*Pl*$_1$	USDA
	4	D-3	RHA 274	*Pl*$_2$/*Pl*$_{21}$	USDA
	1	D-4	DM-2	*Pl*$_5$	USDA
2nd	2	D-5	PM 17	Unknown	USDA
	4	D-6	803-1	Unknown	IFVC
	1	D-7	HA-R4	*Pl*$_{16}$	USDA
3rd	2	D-8	HA-R5	*Pl*$_{13}$	USDA
	4	D-9	HA 335	*Pl*$_6$	USDA
		INRA Proposed Differential Lines[b]			
	1	D-10	Y7Q	*Pl*$_6$-	INRA
4th	2	D-11	PSC8	*Pl*$_2$	INRA
	4	D-12	XA	*Pl*$_4$	INRA
	1	D-13	PSS2RM	*Pl*$_6$/*Pl*$_{21}$	INRA
5th	2	D-14	VAQ	*Pl*$_5$	INRA
	4	D-15	RHA 419	*Pl*$_{Arg}$	USDA
		Supplemental Lines[c]			
			RHA 340	*Pl*$_8$	USDA
			HA 458	*Pl*$_{17}$	USDA
			HA DM 1	*Pl*$_{18}$	USDA
			RHA 468	*Pl*$_{Arg}$	USDA
			TX 16R	Unknown	USDA
			RHA 428	Unknown	USDA
			RNID	*Pl*$_{15}$	Nidera, S.A. (Argentina)

D-1 to D-15, lines of differential set.

Pl indicates *Plasmopara* resistance gene where known.

[a]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000).

[b]Differential set of sunflower lines used for determination of virulence phenotype for isolates of *P. halstedii* proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012).

[c]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines.

[d]Bertero de Romano et al. 2010; Gascuel et al. 2015; B. Hulke, *personal communication*; Liu et al. 2012; Miller and Gulya 1991; Miller et al. 2002; Molinero-Ruiz et al. 2003; Mulpuri et al. 2009; Rahim et al. 2002; Qi et al. 2015; 2016; Vincourt et al. 2012; Zimmer and Kinman 1972.

[e]USDA, United States Department of Agriculture; IFVC, Institute for Field and Vegetable Crops, Yugoslavia; INRA, Institut National de la Recherche Agronomique, France.

Seeds of each line were bleached and germinated on dampened machine cloth on metal trays in the germination chamber at room temperature. When sufficient seed was available, 20 seeds per line were used for virulence screening. Two-ounce disposable plastic cups of seeds from each line to be screened were counted out depending on the number of isolates to be inoculated at one time, with up to ten isolates inoculated in one day. Deionized water was poured into the cups or sprayed onto the seedlings to prevent the roots from drying out. The cups were then drained, if necessary, and placed onto one tray full of differential lines or supplemental lines for each isolate. Two to three sporulated cotyledons per pathogen isolate were added per 100 ml of solution, with approximately 25 ml of spore solution required to cover the seedlings in each cup. A plastic bottle of the spore solution for a particular isolate was shaken multiple times over ten to fifteen minutes prior to pouring the solution over the seedlings in each cup on the isolate tray. Trays were placed in the dark in the mist chambers for a minimum of three hours. Two 38 x 53 x 7.6 cm flats (Kadon Corp., Dayton, OH) were filled about ¾ full with 2 parts washed sand to 1-part perlite by volume for each isolate. The sand/perlite mix was leveled, lightly watered and then pressed to create shallow trenches for each differential or supplemental line. Inoculated seeds were placed root down in the trenches with sterile tweezers, covered with sand and gently watered. Flats were watered daily. Prior to sporulation seed coats were removed, so that cotyledons and true leaves were visible. True leaves were visible on each line after 11-14 days. Flats were misted for 16-48 hours at 100% relative humidity at 16-18°C. Plants were left on a counter to dry completely before rating susceptibility and resistance.

Assessment of virulence on each line was done using a scale proposed by INRA, where SI = susceptible, sporulation on cotyledons and true leaves, SII = abundant sporulation on

cotyledons only, RII = weak sporulation on cotyledons and RI = resistant, no sporulation (Tourvieille de Labrouhe et al. 2012). In this study, a rating of RII was given for sporulation on a few to several plants that was present, but not easily seen from 50 cm away in normal light, all the way to easily visible sporulation of moderate quantity.

In order to express the aggregate virulence phenotype of isolates as races, the internationally accepted triplet code nomenclature was used (Tourvieille de Labrouhe et al. 2000). In the triplet code system, each set of three differential lines is given a numerical value (Table 2.1). The first three lines correspond to the first digit, the second three lines correspond to the second digit and the third three lines correspond to the third digit. Each digit ranges from 0 if all three lines were resistant to 7 if all three lines were susceptible. If a line is resistant, it is given a value of 0. Otherwise, the first line is given a 1, the second line a 2 and the third line a 4. The values for all three lines in the set are then added. For example, the 7 in the 714 is because 1 + 2 + 4 = 7. The 1 for the 2nd digit is because the first line was susceptible and the second two lines were resistant and therefore 0. For the 3rd digit, the first two lines were 0 and the last line was a 4. INRA's proposed two additional sets of three differential lines would bring the total number of digits in the virulence phenotype code to five (Tourvieille de Labrouhe et al. 2012).

Results

Isolates

Throughout this study, 185 total *P. halstedii* isolates were evaluated on the Standard set of nine differential lines, the six INRA proposed differential lines and up to seven supplemental sunflower lines containing additional resistance genes. In 2014, 105 isolates were virulence phenotyped from 62 locations in North Dakota, 13 locations in South Dakota and 6 locations in Minnesota. In 2015, 80 isolates were virulence phenotyped from 59 locations in North Dakota,

12 locations in South Dakota, 5 locations in Minnesota and 4 locations in Nebraska. The number of isolates tested on INRA proposed differential lines and supplemental lines varied depending on seed quality and quantity.

Standard Set of Differential Lines

Virulence was observed on all nine differential lines from the Standard set (Table 2.2), but no one isolate conferred virulence on all nine lines. The Pl_1 gene in RHA 265, differential D-2, was susceptible to all 185 isolates. The Pl_2/Pl_{21} gene cluster in RHA 274, differential D-3, and the Pl_5 gene in DM-2, differential D-4, were susceptible to the majority of the collected isolates at 92% and 75%, respectively. All 14 isolates that were avirulent to the Pl_2/Pl_{21} gene cluster in RHA 274 were virulent on the Pl_6 gene in HA 335. Virulence on lines PM-17 and 803-1, differential lines D-5 and D-6, with unknown genetic resistance, was limited to 8% and 6% of the isolates, respectively. Only one isolate each year was virulent on lines HA-R4 and HA-R5, differential lines D-7 and D-8, with resistance genes Pl_{16} and Pl_{13}. Virulence on the Pl_6 gene, differential D-9, was found for 50.5% of the isolates from 2014 and 42.5% of the isolates from 2015 with an average of 47%.

Based on the current Standard nine *P. halstedii* differential lines, twelve races were found in 2014 and 2015 among isolates from North Dakota, South Dakota, Minnesota and Nebraska (Figure 2.1). In both years, the most common *P. halstedii* races were 714, 710 and 700, comprising 77% of the total races identified. Race 774 was the 4th most frequent race in 2014, while race 314 was the 4th most frequent race in 2015. Three races, 304, 707 and 717, were new to the United States; however, they have previously been identified in France (Virányi et al. 2015).

Table 2.2. Number of *Plasmopara halstedii* isolates collected in 2014 and 2015 in the North Central Great Plains virulent on the Standard set of differential lines.

Differential Set[a]		2014 Isolates Virulent / Isolates Screened	2015 Isolates Virulent / Isolates Screened	Total Isolates Virulent / Isolates Screened	Percent of Isolates Virulent on Differential Line
D-1	Susceptible (MYC 270)	105/105	80/80	185/185	100%
D-2	RHA 265	105/105	80/80	185/185	100%
D-3	RHA 274	101/105	70/80	171/185	92%
D-4	DM-2	83/105	56/80	139/185	75%
D-5	PM 17	10/105	5/80	14/185	8%
D-6	803-1	9/105	3/80	12/185	6%
D-7	HA-R4	1/105	1/80	2/185	1%
D-8	HA-R5	1/105	1/80	2/185	1%
D-9	HA 335	53/105	34/80	87/185	47%

[a]Differential lines according to Tourvieille de Labrouhe et al. (2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*).

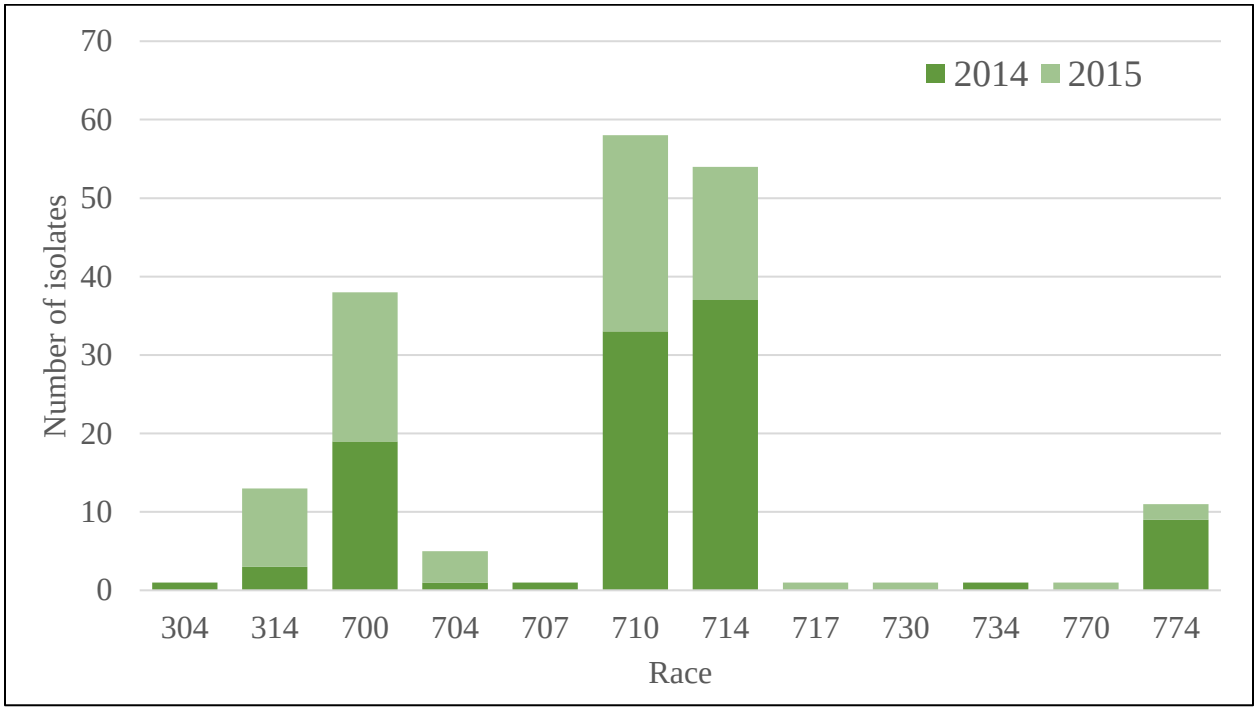

Figure 2.1. *Plasmopara halstedii* races for isolates collected in 2014 and 2015 in the North Central Great Plains using triplet code race nomenclature based on virulence pattern of the Standard set of nine differential lines according to Tourvieille de Labrouhe et al. (2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*).

Proposed INRA Lines

The virulence phenotype of a sub-selection of the 185 isolates collected in 2014 and 2015 was determined using the INRA proposed differential lines, Y7Q, PSC8, XA, PSS2RM, VAQ and RHA 419 (Table 2.3) (Tourvieille de Labrouhe et al. 2012). Virulence was observed on all five of the INRA lines (differential lines D-10 to D-14). The *Pl*$_2$ resistance gene, differential D-11, and the *Pl*$_4$ resistance gene, differential D-12, were susceptible to all or most of the isolates screened at 100% and 86%, respectively. The percent of isolates virulent on the *Pl*$_{6-}$ resistance gene, differential D-10, and the *Pl*$_6$/*Pl*$_{21}$ resistance gene combination, differential D-13, were 49% and 34% respectively. The resistance gene *Pl*$_5$, differential D-14, was the least susceptible to the screened isolates at 7% of the isolates virulent. No virulence was observed on USDA line RHA 419, differential D-15, with the *Pl*$_{Arg}$ resistance gene.

Table 2.3. Number of *Plasmopara halstedii* isolates collected in 2014 and 2015 in the North Central Great Plains virulent on the INRA proposed differential lines.

Differential Set[a]		2014 Isolates Virulent / Isolates Screened	2015 Isolates Virulent / Isolates Screened	Total Isolates Virulent / Isolates Screened	Percent of Isolates Virulent on Differential Line
D-10	Y7Q	27/64	17/26	44/90	49%
D-11	PSC8	65/65	26/26	91/91	100%
D-12	XA	61/65	17/26	78/91	86%
D-13	PSS2RM	18/48	7/26	25/74	34%
D-14	VAQ	1/33	3/26	4/59	7%
D-15	RHA 419	0/105	0/80	0/185	0%

[a]Differential lines according to Tourvieille de Labrouhe et al. (2012) at Institut National de la Recherche Agronomique, France.

Supplemental Differential Lines

Virulence was determined on seven additional lines that have been made available to the public with the exception of RNID, a proprietary line from Argentina, and TX 16R. Seven isolates were found over the two years in North Dakota that were virulent on RHA 340, which

contains the *Pl*$_8$ gene (Table 2.4). Virulence to *Pl*$_8$ by these isolates was confirmed using four other inbred lines containing the same gene, RHA 436, RHA 437, RHA 438 and HA 460, as well as two different years of origin of the RHA 340 seed.

Table 2.4. Number of *Plasmopara halstedii* isolates collected in 2014 and 2015 in the North Central Great Plains virulent on seven supplemental differential lines.

Resistance Gene[a]	Sunflower Line[b]	2014 Isolates Virulent / Isolates Screened	2015 Isolates Virulent / Isolates Screened	Total Isolates Virulent / Isolates Screened	Percent of Isolates Virulent on Differential Line
Pl$_{15}$	RNID	0/66	0/80	0/146	0%
Pl$_8$	RHA 340	2/105	5/80	7/185	4%
Pl$_{17}$	HA 458	0/61	0/80	0/141	0%
Pl$_{18}$	HA DM 1	0/87	0/80	0/167	0%
Pl$_{Arg}$	RHA 468	0/66	0/80	0/146	0%
Unknown	TX 16R	0/84	0/80	0/164	0%
Unknown	RHA 428	15/66	0/0	15/66	23%

[a]Bertero de Romano et al. 2010; Miller and Gulya 1991; Miller et al. 2002; Qi et al. 2015; 2016; B. Hulke, *personal communication*.
[b]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines.

No virulence was found on supplemental lines containing resistance genes *Pl*$_{15}$ (RNID), *Pl*$_{17}$ (HA458) and *Pl*$_{18}$ (HA DM 1). While confirming the pedigree for RHA 468 it was recently discovered that its resistance to downy mildew came from *Pl*$_{Arg}$ in RHA 419; accordingly, like RHA 419, no virulence was found on RHA 468 (B. Hulke, *personal communication*). Supplemental line TX 16R usually had one to three susceptible seedlings out of approximately twenty seedlings for each isolate indicating possible seed contamination. Because RHA 428 was susceptible to 15 of the 66 isolates it was evaluated with in 2014, it was not tested in 2015.

Proposed Race Nomenclature

To express aggregate virulence phenotypes of *P. halstedii* isolates and populations as "races" the Standard set of nine differential lines according to Tourvieille de Labrouhe et al. (2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*), the

INRA proposed differential lines (Tourvieille de Labrouhe et al. 2012) and the most effective three supplemental lines were combined into a single new expanded set (Table 2.5). The differential lines were organized into six groups of three, with each differential given scoring values consistent with the proposed race nomenclature discussed at the 1998 International Sunflower Association Symposium on Sunflower Downy Mildew (Gulya et al. 1998) whereas; the first differential line is given a 1, the second line a 2 and the third line a 4. The values for all three lines in the set are then added and presented as a single number. Using the new proposed differential set, distinctions among several isolates previously named using the Standard differential set (for example, race 710) were observed (Table 2.6). This is particularly true for isolates characterized as races 700, 710 and 714, where they now can be distinguished into two (700600 and 700602), three (710600, 710602 and 710702) and two (714700 and 714710) races, respectively (Table 2.6).

Discussion

The objectives of this study were to evaluate *P. halstedii* virulence phenotype changes in the North Central Great Plains using the current Standard set of nine differential lines and to evaluate INRA proposed differential lines and additional lines containing newer resistance genes for their utility in elucidating additional virulence phenotypes present in the pathogen population. This study was the first to evaluate United States isolates using the Standard set of nine differential lines (Tourvieille de Labrouhe et al. 2000; T. Gulya, *personal communication*), the six new differential lines proposed in 2012 by INRA (Tourvieille de Labrouhe et al. 2012) and additional lines selected with newer resistance genes. The incorporation of additional supplemental lines allowed us to more clearly understand the virulence patterns of *P. halstedii* in the United States, facilitated the first reported identification of virulence to Pl_8 and provided data

Table 2.5. Proposed race nomenclature that incorporates the nine Standard differential lines[a], the differential lines proposed by INRA[b] and three supplemental lines evaluated in this study of *Plasmopara halstedii* isolates collected in 2014 and 2015 in the North Central Great Plains.

Triplet Code		**Differential Set**		**16 *Plasmopara halstedii* Aggregate Virulence Phenotypes**															
Digit	Value	No.	Sunflower Line	304300	314300	700600	700602	704710	707710	710600	710602	710702	714700	714710	717710	730600	734710	770620	774730
1st	1	D-1	Mycogen 270	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	2	D-2	RHA 265	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	4	D-3	RHA 274	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2nd	1	D-4	DM-2	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S
	2	D-5	PM 17	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
	4	D-6	803-1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
3rd	1	D-7	HA-R4	R	R	R	R	R	S	R	R	R	R	R	S	R	R	R	R
	2	D-8	HA-R5	R	R	R	R	R	S	R	R	R	R	R	S	R	R	R	R
	4	D-9	HA 335	S	S	R	R	S	S	R	S	R	S	S	S	R	S	R	S
4th	1	D-10	Y7Q	S	S	R	R	S	S	R	S	S	S	S	S	R	S	R	S
	2	D-11	PSC8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	4	D-12	XA	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5th	1	D-13	PSS2RM	R	R	R	R	S	S	R	S	R	R	S	S	R	S	R	S
	2	D-14	VAQ	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
	4	D-15	RHA 419	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
6th	1	D-16	RNID	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	2	D-17	RHA 340	R	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R
	4	D-18	HA 458	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

[a]Differential lines according to Tourvieille de Labrouhe et al. (2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*).
[b]Differential lines according to Tourvieille de Labrouhe et al. (2012) at Institut National de la Recherche Agronomique, France.

for a discussion at the 19th International Sunflower Conference in Edirne, Turkey in 2016 in which revisions to the current and INRA proposed race nomenclature and the addition of three new differential lines were discussed by scientists from France, Argentina and the United States.

Table 2.6. Current race nomenclature (three digits) and proposed race nomenclature (six digits) using aggregate virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 and 2015 in the North Central Great Plains.

Race Based on Standard Differential Lines[a]	2014 Isolates	6-Digit Aggregate Virulence Phenotype[b]	2015 Isolates	6-Digit Aggregate Virulence Phenotype[b]
304	1/1	304300	---	---
314	0/3	---	8/10	314300
700	4/19	700600	0/19	---
	1/19	700602	1/19	700602
704	1/1	704710	2/4	704710
707	1/1	707710	---	---
710	11/33	710600	3/25	710600
	1/33	710602	3/25	710602
	---	---	1/25	710702
714	1/37	714700	---	---
	5/37	714710	2/17	714710
717	---	---	1/1	717710
730	---	---	1/1	730600
734	1/1	734710	---	---
770	---	---	1/1	770620
774	1/9	774730	2/2	774730

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.
[b]Proposed race nomenclature for the six-digit aggregate virulence phenotype includes the addition of six INRA proposed lines (Institut National de la Recherche Agronomique, France, Tourvieille de Labrouhe et al. 2012) as digits four and five and three supplemental lines as digit six. Specifically, these are sunflower lines Y7Q, PSC8 and XA for digit four, sunflower lines PSS2RM, VAQ and RHA 419 for digit five and sunflower lines RNID, RHA 340 and HA 458 for digit six.

In this study, a total of 22 lines were evaluated as differential lines. The results of this evaluation demonstrate that the inclusion of differential lines not represented in the original Standard set of nine differential lines is critical. While the addition of lines in the proposed INRA set is useful to differentiate virulence, the addition of three lines evaluated in the study is also necessary for accurate assessment of virulence into the future. In addition, results from this

study demonstrated that not all of the 22 differential lines evaluated were equally useful in differentiating virulence in the North Central Great Plains.

Virulence phenotypes as determined by the nine lines in the original standard differential set are still useful for consistency of race nomenclature. However, not all differential lines were particularly useful for differentiating virulence in the *P. halstedii* population in the United States. For example, all of the 185 isolates used in this study confer virulence on differential D-2 (RHA 265) and differential D-3 (RHA 274), and nearly all confer virulence on differential D-4 (DM-2). Although this is not surprising, as each contains resistance genes released many decades ago, it reduces their usefulness in future studies and those resistance genes are no longer actively bred into hybrids for use in the United States. However, limited virulence was observed on differential lines D-5 (PM 17), D-6 (803-1), D-7 (HA-R4) and D-8 (HA-R5). This is possibly because the genes they conferred are not thought to be incorporated into commercial hybrids. One of the most important differential lines in this set is differential D-9, which contains the Pl_6 gene. This resistance gene was widely deployed in the United States, and virulence was first found in 2009 in Bottineau County, North Dakota (Gulya et al. 2011). Approximately half of the isolates identified in this study conferred virulence on this gene. However, the differential is still useful and is widely used and pyramided with other genes.

Based only on the current set of nine Standard *P. halstedii* differential lines, twelve races were found in 2014 and 2015 in isolates from North Dakota, South Dakota, Minnesota and Nebraska. In both years, the most common *P. halstedii* races were 714, 710 and 700, comprising 77% of the total races identified. Race 774, a race which confers virulence on 7 of 9 differential lines, was the 4th most frequently identified race in 2014, while race 314 was the 4th most frequent race in 2015. Three races, 304, 707 and 717, were identified in this study for the first

time in the United States; however, they have previously been identified in France (Virányi et al. 2015). Races did not appear to be clustered in any area.

Given the international nature of commercial sunflower production, it was prudent to evaluate the six differential lines proposed by INRA (Tourvieille de Labrouhe et al. 2012) for use in the United States. Virulence was observed on all five of the INRA lines used as Differential lines D-10 to D-14 (Y7Q, PSC8, XA, PSS2RM, VAQ), and was particularly common on the first three differential lines, where the majority of isolates used in this study were found to be virulent. However, these lines still add value to the differential set. Differential D-10, can be useful in North America to distinguish races previously only characterized as 710. One isolate found in this study conferred virulence on Y7Q which has the Pl_{6-} gene giving it an aggregate virulence phenotype of 710702, allowing distinction between 710702 and 710602. In the North Central Great Plains, differential D-11 was not found necessary as all of the isolates tested were virulent on it. However, in France, differential D-11 distinguishes between aggregate virulence phenotypes 30410 and 30430. Differential D-12, XA, is a unique gene, Pl_4, but most of the isolates overcome it. Differential D-13, PSS2RM, is a new gene combination of Pl_6/Pl_{21} that is only slightly more effective than differential D-9 with just the Pl_6 gene. One isolate found in this study conferred virulence on D-13 giving it an aggregate virulence phenotype of 714710, allowing distinction between 714700 and 714710. Differential D-14, VAQ, is not the only differential to have the Pl_5 gene, but it has a different reaction than the Standard differential D-4 which has been used in the United States, DM-2 with genes Pl_5, Pl_{11} and Pl_{12}. This is the first time isolates with the virulence phenotypes 734 and 770 have been evaluated with these proposed differential lines as these races have not been found in Europe. With the additional differential lines in the INRA set their aggregate virulence phenotypes were 734710 and 770620.

The most useful line for determining pathogen virulence into the future is likely differential D-15, RHA 419, which contains the Pl_{Arg} gene, which is likely being incorporated into commercial germplasm.

Prior to conducting the survey, the Standard set of nine differential lines did not fully represent the resistance genes already deployed and the six INRA proposed differential lines only added one new resistance gene Pl_{Arg}, RHA 419, that was being deployed in the United States (Tourvieille de Labrouhe et al. 2000; 2012). Therefore, virulence was determined on seven supplemental lines that have been made available to the public with the exception of RNID, a proprietary line from Argentina, and TX 16R. One of the resistance genes was $Pl_{8,}$ which was incorporated into inbred lines RHA 340, RHA 436, RHA 437, RHA 438 and HA 460. Using the proposed differential D-16, RHA 340, virulence to the Pl_8 resistance gene was found in seven isolates collected in the study. As this was the first identification of virulence to the gene in the world (Trojanová et al. 2017), greenhouse seedling tests on four additional inbred lines with the Pl_8 resistance gene were used to confirm that virulence existed as well as different years of origin of the RHA 340 seed. Additionally, one of the two isolates from 2014 that conferred virulence on the Pl_8 resistance gene was tested in the field with and without fungicidal seed treatments to confirm virulence (Humann et al. 2016). No virulence was found on supplemental lines containing resistance genes Pl_{15} (RNID), Pl_{17} (HA458) and Pl_{18} (HA DM 1). While confirming the pedigree for RHA 468 it was recently discovered that its resistance to downy mildew came from Pl_{Arg} in RHA 419 (B. Hulke, *personal communication*); accordingly, like RHA 419, no virulence was found on RHA 468. Supplemental line TX 16R usually had one to three susceptible seedlings out of approximately twenty seedlings for each isolate indicating

possible seed contamination. Because RHA 428 was susceptible to 15 of the 66 isolates it was evaluated with in 2014, it was not tested in 2015.

In order to more easily discuss the new virulence to the Pl_{15} gene in Argentina and the Pl_8 gene in the United States, it was proposed at the International Sunflower Conference in Turkey in 2016 that these two genes become differential lines D-16 and D-17, respectively (A. Bertero de Romano and M. Sposaro, *personal communication*). The sunflower inbred line chosen for differential D-16 was IR101DMR which had been further selected from RNID and the sunflower inbred line chosen for differential D-17 was RHA 340 *(*M. Sposaro, *personal communication*). Differential D-18, HA 458, with differential gene Pl_{17} was added out of necessity as differential lines must be added in sets of three and it was the oldest gene to be determined that was not already a differential line. A new susceptible check, differential D-1, was proposed to be HA 821 (Terres Inovia, France, *unpublished data*). Sunflower line PSS2RM, one of the INRA proposed differential lines was replaced with 83HR4RM for differential D-13 with the same Pl_6/Pl_{21} resistance gene (Terres Inovia, France, *unpublished data*). Sunflower downy mildew continues to be an important topic of concern at the International Sunflower Conference meetings every four years. Hopefully, these new differential lines will be evaluated internationally, so they can be endorsed internationally at some point in the future.

One of the isolates conferring virulence to the Pl_8 gene was collected from a volunteer sunflower in a soybean field in 2014. The field had likely not yet been treated with an herbicide, and consequently, was so filled with sunflowers that it was not obvious until close examination if the planted crop was soybean or sunflower. The finding of this virulent isolate in a field full of diseased volunteer plants indicates the importance of controlling volunteer sunflowers as part of sunflower downy mildew management. Volunteer sunflowers are the perfect source for spread

of the pathogen since volunteer sunflower seeds are never treated with a fungicide and sequential "production" of sunflower in a field increases the selection pressure on the resistance genes deployed to control disease.

To evaluate the selection pressure on the pathogen in sunflower nurseries and sunflower disease trials as compared to surveyed fields, a comparison was made of the percentage of isolates virulent on the Pl_6 gene between commercial fields surveyed and nurseries and experimental plots surveyed. The percentage of isolates virulent on the Pl_6 gene from both subsets was exactly 47%. This, and the fact that all isolates virulent on the Pl_8 resistance gene were found in surveyed fields indicates that conditions in nurseries and experimental plots are not providing more selection pressure for *P. halstedii* than growers' fields in the Northern Great Plains. A somewhat similar result was suggested in the sunflower rust pathosystem (P*uccinia helianthi*), where *P. helianthi* isolates were obtained over a similar time period (two years) and from similar hosts (commercial fields, experimental trials, volunteers, wilds) (Friskop 2015). Friskop (2015) observed no perceived increase in diversity among host collections in the U.S. Northern Great Plains, but suggested that a higher level of virulence diversity may exist in the sunflower seed production areas in California. This study did not include any isolates from the seed production area in California, but additional work to examine the virulence diversity of *P. halstedii* in that region may be interesting.

The sunflower industry in the United States is fortunate to have at least four resistance genes that are believed to be completely effective, Pl_{Arg}, Pl_{15}, Pl_{17} and Pl_{18}, as well as three resistance genes, Pl_8, Pl_{13} and Pl_{16}, for which little virulence has been observed. The seven isolates conferring virulence to Pl_8 were unable to overcome many more common resistance genes, including Pl_6. Isolates that confer virulence on the Pl_{15} resistance gene have been found

in Argentina (Trojanová et al. 2017). A new downy mildew resistance gene, *Pl*$_{19}$, in confection sunflower was not evaluated as part of this study, but five *P. halstedii* isolates from this study were provided to confirm resistance (Zhang et al. 2016). However, the number of resistance genes that have been overcome by the pathogen in the North Central Great Plains is alarming. This is particularly true as sunflower in the United States is considered a minor hectarage crop, typically planted only once every four years to a field, and was only commercially cultivated for the last five decades. Consequently, it is imperative to protect the new resistance genes as much as possible, and frequently monitor the pathogen for virulence changes. Differential lines proposed in this manuscript, which follows up on discussions during the International Sunflower Conference in 2016, advance the ability of scientists internationally to monitor virulence changes more effectively and breed for resistance to downy mildew.

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CHAPTER 3. EVALUATION OF *PLASMOPARA HALSTEDII* SENSITIVITY TO AZOXYSTROBIN

Introduction

Downy mildew, caused by the biotrophic oomycete pathogen *Plasmopara halstedii* (Farl.) Berl. and de Toni, is an economically important disease in cultivated sunflowers, *Helianthus annuus* L. Downy mildew occurs sporadically within a field, and is dependent on free-soil moisture and cool temperatures following planting (Zimmer 1975). Fungicidal seed treatments have been widely used for protection against downy mildew (Gulya et al. 1997; Markell et al. 2016). The phenylamide fungicide seed treatments metalaxyl (Allegiance, Gustafson, Plano, TX) and mefenoxam (Apron-XL, Novartis, Greensboro, NC) were an effective management tool between 1985, when metalaxyl was released, until their widespread failure in 1998 and 1999 when *P. halstedii* developed insensitivity (Gulya 2000; Gulya et al. 1999). The first reported insensitivity of *P. halstedii* isolates to metalaxyl was in 1995 in France (Albourie et al. 1998). Insensitivity to these fungicides had already been reported in the oomycete pathogens *Plasmopara viticola, Bremia lactucae* and *Pseudoperonospora cubensis* causing downy mildew of grapes (*Vitis vinifera* L.), lettuce (*Lactuca sativa*) and cucurbits (Cucurbitaceae), respectively, and late blight (*Phytophthora infestans*) and pink rot (*Phytophthora erythroseptica)* of potatoes (*Solanum tuberosum* L.), so it was not altogether unexpected (Abu-El Samen et al. 2005; Gisi and Sierotzki 2015; Grünwald et al. 2006; Heaney et al. 2000). However, it was thought that the risk of resistance development for metalaxyl and mefenoxam would remain low because *P. halstedii* was a soilborne pathogen; therefore, the fungicide was applied only once per year as a seed treatment (Gulya et al. 1999).

Following the discovery of metalaxyl and mefenoxam insensitivity, two additional fungicides were found to have varying levels of efficacy: azoxystrobin and fenamidone (Gulya 2002). Both fungicides are considered to be fungistatic rather than fungicidal at the rates tested (Gulya 2002). Both azoxystrobin and fenamidone are in the FRAC 11 fungicide group, the quinone outside inhibitors (QoIs), and are classified as having a high risk of resistance development (FRAC 2016). Azoxystrobin was labeled for use in the United States in 1997 as Heritage (Syngenta Crop Protection, Greensboro, NC) on turf and Abound (Syngenta Crop Protection, Greensboro, NC) on fruit, nuts and vegetables (Uttley 2011). The first known use of azoxystrobin on farm fields in the states of North Dakota, Minnesota, Nebraska and Wisconsin was after July 30, 1998 on potatoes after an emergency use label was granted (Pasche et al. 2004). Azoxystrobin was labeled for use on sunflowers as Protégé (Gustafson, Plano, TX) in 2003 and later as Dynasty (Syngenta Crop Protection, Greensboro, NC) (Bradley 2003). Fenamidone, first sold in 2001, was released as Idol (Bayer Crop Science, Research Triangle Park, NC) and its label was amended to include sunflowers in October of 2007 (Kish 2007). Fenamidone was withdrawn in 2012 leaving only azoxystrobin, which was labeled only for suppression of downy mildew of sunflowers.

Azoxystrobin, like the other QoIs, targets respiration in fungal and fungal-like mitochondria by binding to an enzyme on the quinone outside site of the cytochrome bc1 complex, preventing the transfer of electrons between cytochrome b and cytochrome c1 (Fernandez-Ortuño 2008). To date, three mutations in the cytochrome b gene, which differentially effect pathogen sensitivity, have been found. These amino acid substitutions either slow or prevent binding and are considered to be qualitative selection (FRAC 2014). G143A, the most common substitution, is a change from glycine to alanine at position 143 and causes

complete failure of the fungicide in the field (FRAC 2014). With the G143A mutation, the resistance factor (RF=EC_{50} of the resistant strain/EC_{50} of the sensitive strain) usually exceeds 100 and can be several hundred (FRAC 2014). F129L, a change from phenylalanine to leucine at position 129, and G137R, a change from glycine to arginine at position 137, cause reduced sensitivity that is still controlled by the fungicide (FRAC 2014). As of 2012, FRAC has confirmed field resistance in 25 pathogens with the G143A mutation, five pathogens with the F129L mutation, two pathogens with both the G143A and the F129L mutations and one pathogen with all three mutations (FRAC 2012).

As in the case of metalaxyl and mefenoxam, azoxystrobin applied as an annual seed treatment has a lower risk of resistance development than azoxystrobin applied multiple times during a season as a foliar fungicide (Russell 2003). However, the related foliar oomycete pathogens, *P. viticola, P. cubensis* and *Pythium aphanidermatum* have developed insensitivity to azoxystrobin (FRAC 2012; Genet et al. 2006; Gisi et al. 2000; Ishii et al. 2001). *Plasmopara viticola* has both the G143A and the F129L mutations, *P. cubensis* has the G143A mutation and *P. aphanidermatum* has the F129L mutation (FRAC 2012). A baseline to monitor resistance development to azoxystrobin has not been developed.

A baseline for how a pathogen responds to a chemical is calculated using pathogen isolates collected prior to the use of a fungicide or from locations where it is known that the fungicide was never applied. If the pathogen can be cultured on artificial medium, then pathogen growth can be measured on fungicide-amended media (Russell 2003). Assays of spore germination and germ tube elongation may be conducted depending on the fungicide and pathogen (Russell 2003). Another factor to be considered is the use of single spore isolates or a population of spores (Russell 2003). Isolates are tested at a range of fungicide concentrations to

determine a mean concentration that would produce 50% inhibition of the pathogen, Effective Control (EC_{50}), when compared to a non-fungicide control. In vitro testing to establish a baseline for *P. viticola*, an obligate oomycete pathogen, can be done by collecting leaves with oospores, overwintering them in the vineyard and filtering the oospores from the leaves before conducting oospore germination assays on fungicide-amended water agar (Corio-Costet 2015). In vivo testing to establish a baseline for an obligate oomycete pathogen requires the use of detached plant parts as is the case for most foliar pathogens or whole plants as is necessary for *P. halstedii*, primarily a root pathogen (Russell 2003). This requires applying the fungicide and then inoculating the plant with spores. With obligate pathogens, it is expected that cost, time and space will limit the number of pathogen isolates and fungicide concentrations tested (Russell 2003).

Given labor and cost constraints, few fungicide sensitivity studies have been conducted on *P. halstedii*. Similarly, methods vary widely, a reflection of the complexity associated with an obligate, root-infecting oomycete. In a study to demonstrate that loss of efficacy of metalaxyl in France, sensitivity was evaluated by treating seeds, inoculating with a whole seedling immersion technique, growing plants in a growth chamber and visually scoring for systemic infection (Albourie et al. 1998). In a study in the United States confirming loss of efficacy of metalaxyl, seeds were treated and planted, flats were drenched with a spore suspension, plants were grown out on water-cooled benches in the greenhouse and visually evaluated for systemic infection (Gulya 2000). In the first of two greenhouse studies in Spain confirming loss of efficacy of both metalaxyl and mefenoxam, seeds were treated and planted prior to being drenched with a spore suspension and in the second study treated seedlings were inoculated using the whole seedling immersion technique and then planted, grown and evaluated for

systemic infection (Molinero-Ruiz et al. 2005). Two studies in Germany used 5 mm diameter leaf discs cut from three-week old plants in fungicide-amended water, in which leaf discs were inoculated with 5,000 sporangia, kept for 14 days in a cabinet at 16ºC with a 14-hour photoperiod and evaluated for sporulation (Rozynek and Spring 2001; Spring et al. 2006). Results of this study confirmed resistance in a French isolate, but not a German isolate that showed resistance in the field (Rozynek and Spring 2001; Spring et al. 2006). Multiple fungicide efficacy trials have also been completed with *P. halstedii* using similar methodology to the fungicide sensitivity studies. To the best of our knowledge no studies have been done to establish a sensitivity baseline to a fungicide labeled only to suppress infection by *P. halstedii*.

A discriminatory dose is usually set after fungicide resistance is confirmed to classify isolates as sensitive, reduced-sensitive or resistant. It is based on a fungicide concentration that nearly (EC_{90}) or completely (Minimum Inhibitory Concentration) inhibits sensitive isolates, while resistant isolates have greater than 50% growth (Wise et al. 2009; Corio-Costet 2015). In studies where a discriminatory dose was set to detect resistance where resistance had not been previously determined, discriminatory concentrations were selected from near the EC_{50} value (Mondal et al. 2005). Later, when resistance was found, a new higher discriminatory dose was set and used where resistant isolates had greater than 50% growth (Wong et al. 2007). In another study, a discriminatory dose was established based on the EC_{50} of 31 isolates to screen 282 isolates and the one sensitive isolate out of 282 isolates was later exposed to a range of fungicide concentrations to confirm sensitivity (Lehner et al. 2015).

This study was conducted at the request of the sunflower industry and stakeholders, due to concerns of high incidence in downy mildew occurring in fields treated with azoxystrobin. Given that azoxystrobin is only suppressive, the high levels of downy mildew some growers and

stakeholders were experiencing could be facilitated by a very favorable environment or the development of fungicide resistance. As such, evaluation of fungicide efficacy in a controlled study was critical. Therefore, the objectives of this study were to establish baseline sensitivity to azoxystrobin and determine if *P. halstedii* had developed fungicide resistance after eleven years of use of Dynasty as a sunflower seed treatment.

Materials and Methods

Plasmopara halstedii isolates

To conduct a fungicide sensitivity study, *P. halstedii* isolates that were not exposed to the fungicide in nature were needed. Over a period of several months, 62 isolates initially collected between 1981 and 1994 were tested for viability. The alphanumeric code on each cryotube or scintillation vial was traced back to the original logbooks to confirm that the isolate came from the United States during the appropriate time period. To produce fresh pathogen material, about 100 pre-germinated seedlings of the susceptible genotype, USDA inbred HA 89, were placed in labeled four-ounce disposable plastic cups in 50 ml of a 10 mM calcium chloride solution. Scintillation vials or cryotubes containing sporulated, desiccated cotyledons or vacuumed air-dried spores were removed from storage in a -80ºC freezer. Selected vials or cryotubes were heat shocked in warm water around 38°C for one to two minutes. Spores or cotyledons were stirred into the germinated seedlings and then incubated in the dark at 16-18ºC for at least five hours. The inoculated seedlings were planted in row in large flats containing a blend of sand and perlite and then were covered with a light layer of sand. After eleven days in the greenhouse, plants were induced to sporulate by placing them in a dark mist chamber at 16-18ºC for 16-48 hours. Successful sporulation ranged from just a few to nearly all of the plants. Flats with no or poor sporulation were returned to the greenhouse for an additional week before misting them

again just in case disease development was slow. Multiple samples of an isolate were inoculated separately at first. Then, all samples remaining for an individual isolate were bulked together.

A total of 24 out of 62 pathogen isolates (39%) were revived from a total of 92 scintillation vials and many cryotubes of desiccated spores and cotyledons collected in the United States prior to the first use of azoxystrobin. Only 21 of the isolates were used in the azoxystrobin studies, since one of these isolates was from 1981 and it did not sporulate well on HA 89 or the susceptible hybrid, Mycogen 8N358CL, and the other two isolates came from trials in Enderlin, ND and Grandin, ND where three isolates had already been revived at each location. The viable pre-1997 isolates which were initially collected between 1985 and 1994 originated from North Dakota, Minnesota, Wisconsin, Kansas and Texas (Figure 3.1).

To successfully evaluate current sensitivity to azoxystrobin, isolates were collected during 2014 and 2015 surveys of downy mildew prevalence and incidence in North Dakota and northern South Dakota (Chapter 2). Additional isolates were sent in both years by personnel from USDA-ARS, state Extension services and seed companies. Isolates for this evaluation were from North Dakota, South Dakota, Minnesota and Nebraska (Figure 3.1). To increase pathogen material for these isolates, the same techniques as described above were used, but fewer seeds were started and smaller flats were used since these isolates had been in a -80ºC freezer for much less time.

Development of a Discriminatory Dose

To determine baseline sensitivity to azoxystrobin, three isolates obtained in the United States prior to 1997 and three isolates from 2015 were selected for the discriminatory dose concentration study. The three pre-1997 isolates were from Wisconsin (1991 Prescott #2084 #113 R-line), North Dakota (1992 Enderlin #2 305B) and Minnesota (1993 Glyndon #2341) and

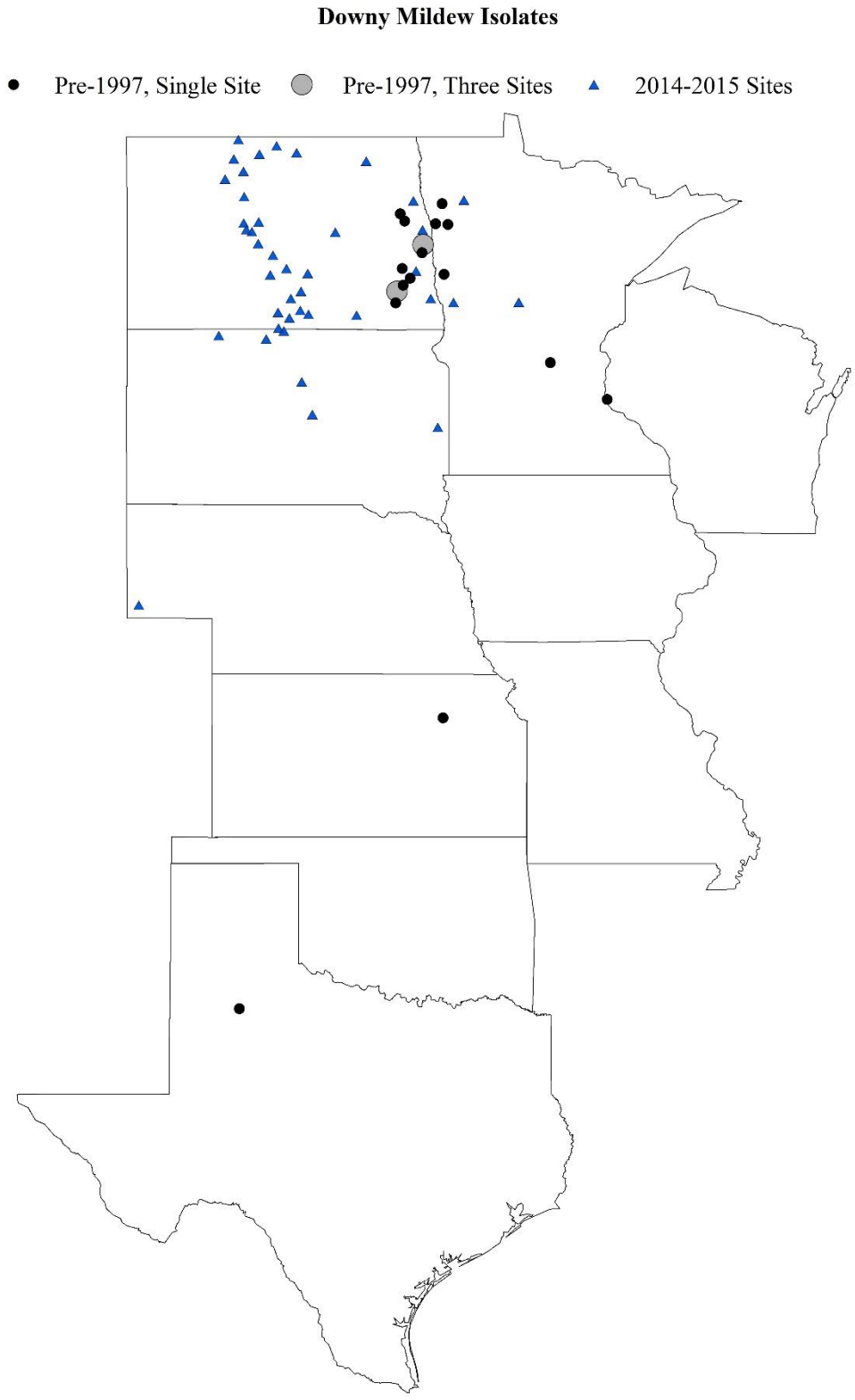

Figure 3.1. Locations of *Plasmopara halstedii* isolates used to establish baseline sensitivity to azoxystrobin with isolates collected prior to 1997 and evaluate sensitivity of isolates collected during 2014 and 2015.

were all collected prior to Protégé or Dynasty being used in the United States. The three 2015 isolates were selected from fields in North Dakota (28C, 29A and 54A) with the highest downy mildew field incidence as observed in a two-year survey (Chapter 1).

To establish a discriminatory dose, three trials were conducted. For each trial of the discriminatory dose concentration study, the six pathogen isolates were arranged in a randomized complete block design with three replications of 36 seeds in the same greenhouse room. The isolates and fungicide concentrations were randomized across each block. Susceptible nontreated oilseed sunflower, Mycogen 8N358CL, was treated with a range of concentrations of a commercial formulation of Dynasty obtained from Syngenta to determine a concentration at which pathogen isolates would be expected to infect 50% of the seedlings, Effective Concentration (EC_{50}). Seed treatment colorant was donated by Chromatech Inc. (Canton, MI). In the first trial, a very wide range of concentrations of azoxystrobin were tested to ensure complete control was achieved: 0, 0.25, 2.5, 25, and 250 µg active ingredient (ai)/seed. Based on the results of the first trial, the concentrations in the second trial, were narrowed to: 0, 2.5, 5, 10 and 20 µg ai/seed. The first two trials were started in the greenhouse and moved for the three-day inoculation period to the much cooler mist chamber room because of the high outdoor temperatures. The third trial was conducted completely in the greenhouse using the same narrowed range of azoxystrobin concentrations used in the second trial.

To conduct discriminatory dose trials, seeds were planted in flats and then put in the greenhouse and inoculated using the soil-drench method for determining fungicide efficacy (Gulya 2002). Hybrid seed Mycogen 8N358CL was used as the downy mildew susceptible line. A washed sand and perlite mix at a 3:1 ratio by volume was used. The sand and perlite blend was put in doubled 26 cm square inserts (T.O. Plastics, Clearwater, MN) with extra drainage

holes and placed in 27 x 54 cm standard web flats (T.O. Plastics, Clearwater, MN). These inserts were filled with approximately the same amount of sand/perlite and leveled to about ¾ full, gently watered and pressed with a 36-peg dibble board. On the first day of the trial, seeds were placed in the holes and covered with approximately 5 mm of pure sand. Flats were watered well after planting. Seeds that were on top of the sand after watering were pushed down. Flats were kept in a greenhouse with a 16 hour photoperiod at approximately 18°C average air temperature. After two days, the flats were moved from the greenhouse to the mist chamber where they could be kept below 22ºC, optimal for inoculation.

Immediately prior to inoculation, flats were separated into six groups and moved to their own shelves on a cart. This ensured that no cross contamination among isolates would occur by leaking, water splash or water uptake during inoculation. On the third, fourth and fifth day after planting, when radicles were one to two cm long and after the morning watering, each insert was evenly inoculated using a Centrospray rubber bulb fitted with a multi-hole spray nozzle. A concentration of 20000 to 40000 spores ml^{-1} in 100 ml of a 10 mM calcium chloride solution was applied. Inserts were watered at least six hours after inoculating. On the fifth day prior to the afternoon watering, flats were moved back to the greenhouse.

On the fourteenth day, plants were placed in a mist chamber by isolate for 48 hours to induce sporulation so plants could be evaluated for infection. The walls and door of the mist chamber and the seedlings were sprayed with a fine mist of water to create 100% relative humidity. The mist chamber room was kept dark at a temperature of 16-18°C. After sporulation, each isolate was separated from other isolates for several hours until dry. Seedlings were examined by flashlight and if there was any sporulation on the seedling it was considered infected.

To calculate mean EC_{50} values for each isolate, percent infection at each concentration by replicate was adjusted relative to the percent infection of the inoculated nontreated seed. The formula used to calculate percent infection for the treated flats was 100 - (((% infection nontreated flat - % infection treated flat) / % infection nontreated flat) x 100) by isolate for each concentration in the replicate. These percentages were regressed against the logarithm of the azoxystrobin concentration using SAS version 9.4 (SAS Institute, Cary, NC) and the EC_{50} was estimated by interpolation to provide mean concentrations for each replicate of each isolate. Each trial was analyzed separately using SAS to test for homogeneity of variance using the general linear model procedure to conduct an analysis of variance (ANOVA) on the mean EC_{50} to determine whether or not the trials could be combined. Mean EC_{50} values by isolate were determined using SAS's least significant difference test at $\alpha = 0.05$ to separate means.

Evaluation of Pathogen Sensitivity

To evaluate *P. halstedii* sensitivity to azoxystrobin, the 18 remaining isolates collected prior to 1997 were used and 39 isolates were selected from isolates collected in 2014 and 2015 for a total of 57 isolates. These isolates were selected proportionally based on the total number collected in each state and then based on geographical dispersion in the states. Twenty-nine isolates originating from North Dakota, six from South Dakota, three from Minnesota and one from Nebraska. Based on the results of the discriminatory dose study, a concentration of 10 ug ai/seed was selected to screen these 57 isolates. At this dose, infection for most isolates in the pathogen sensitivity study would be expected to be between 10 and 30%, based on adjusted percent infection results from the 10 ug ai/seed concentration from the third discriminatory dose trial. Two isolates from North Dakota used in the discriminatory dose study were selected as

reference isolates for the pathogen sensitivity study namely; Enderlin #2 305B from 1992 and 54A from 2015. These isolates were repeated in each pathogen sensitivity trial.

The same methodology for producing fresh pathogen material, planting, inoculation and sporulation was used in the pathogen sensitivity study. In the first set of four trials, 17 isolates were tested per greenhouse and in the last three sets of trials 16 isolates were tested per greenhouse. Each isolate was tested in two greenhouse rooms in a split plot arrangement with subplots of 0 and 10 µg ai/seed with three replications of 36 seeds. The treated and nontreated flats were randomized first and then the isolates were randomized across each block. To calculate mean percent infection values at 10 ug ai/seed for each isolate percent infection by replicate was adjusted relative to the percent infection of the inoculated nontreated seed.

For each trial, the two reference isolates were included for an "assay reproducibility" calculation used by Wong and Wilcox (2002) to generate approximate limits for 95% confidence intervals. Trials in each greenhouse were analyzed separately using SAS version 9.4 to test for homogeneity of variance using the mixed model procedure for split plot RCBD to determine whether or not the trials could be combined. Trials in each greenhouse were analyzed separately using SAS to test for homogeneity of variance using Levene's and Brown and Forsythe's tests. Data was transformed in Excel by using the formula =SQRT (adjusted % infection+0.5) to modify the data due to the violations of assumptions of normality and heterogeneity of variance. Trials in each greenhouse were analyzed separately again using Levene's and Brown and Forsythe's tests for homogeneity of variance and using the general linear model procedure to conduct an analysis of variance (ANOVA) on the mean percent infection to determine whether or not the trials could be combined. To detect differences in the means of the pre-1997 and 2014-2015 groups of isolates, the treated flats were compared using a folded F-test to check for

homogeneity of variances and the pooled method t-test procedure at the 95% level of confidence using SAS.

Results

Development of a Discriminatory Dose

Mean EC_{50}'s ranged from 6.6 to 9.3 ug ai/seed for trial 1, 4.9 to 10.8 ug ai/seed for trial 2 and 5.2 to 7.7 ug ai/seed for trial 3 (Table 3.1). EC_{50} values from the three trials were not combined as the three trials were significantly different. This was not unexpected as fungicide concentrations and inoculation locations varied. Therefore, the results of the least significant difference test were not used. The discriminatory dose for pathogen sensitivity was set at 10 ug ai/seed. This concentration was tested in the discriminatory dose study and adjusted percent infection at this dose was expected to be between 10 and 30% based on results from the third trial which was conducted completely in the greenhouse like the pathogen sensitivity study. Percent infection at the 5 ug ai/seed concentration was too high. An average of the isolate means for the third trial, 6.9%, was also considered, however the discriminatory dose is supposed to nearly or completely control sensitive isolates, and this was the expected outcome at 10 ug ai/seed.

Evaluation of Pathogen Sensitivity

To evaluate the 57 pre-1997, 2014 and 2015 *P. halstedii* isolates for sensitivity to azoxystrobin, mean adjusted percent infection was determined for each isolate using the discriminatory dose concentration of 10 ug ai/seed. While adjusted percentages for most of the treated flats were in the expected range of 10 to 30% infection, this dose resulted in many treated flats with no or few downy mildew infected seedlings contributing to the difficulty in analyzing this data. One inoculated control flat for Enderlin #2 102A from 1992 was poorly infected

Table 3.1. EC_{50} values and means for the three discriminatory dose trials in ug ai/seed.

Isolate[a]	Rep 1	Rep 2	Rep 3	Mean	St Dev	CV
			Trial 1[b]			
28C	7.6	7.9	9.5	8.4	1.0	12.1
29A	5.7	7.9	7.2	6.9	1.1	16.3
54A	7.6	9.1	9.8	8.8	1.1	12.8
305	7.9	11.4	8.6	9.3	1.9	19.9
2084	7.7	8.2	3.9	6.6	2.3	35.4
2341	6.8	8.0	6.9	7.2	0.7	9.1
			Trial 2[c]			
28C	12.2	11.4	8.0	10.5	2.2	20.9
29A	10.4	12.9	9.1	10.8	1.9	17.5
54A	8.5	12.6	8.0	9.7	2.4	25.7
305	5.7	4.5	4.5	4.9	0.7	14.0
2084	10.1	6.3	6.5	7.6	2.1	28.1
2341	7.4	6.2	5.9	6.5	0.8	12.4
			Trial 3[c]			
28C	7.2	7.7	5.0	6.6	1.4	21.6
29A	7.4	7.2	6.6	7.0	0.4	6.1
54A	7.0	7.5	8.6	7.7	0.8	10.9
305	4.1	6.1	5.5	5.2	1.0	19.0
2084	4.8	6.1	7.3	6.1	1.3	20.8
2341	7.5	9.5	5.6	7.5	2.0	26.0

[a]28C, 29A and 54A were from plants in North Dakota in 2015 from fields with the highest downy mildew field incidence as observed in a two-year survey and 305, 2084 and 2341 were from stored isolates Enderlin, ND #2 305B from 1992, Prescott, WI #2084 #113 R-line from 1991 and Glyndon, MN #2341 from 1993.
[b]EC_{50} values based on azoxystrobin concentrations of 0, 0.25, 2.5, 25, and 250 µg ai/seed.
[c]EC_{50} values based on azoxystrobin concentrations of 0, 2.5, 5, 10 and 20 µg ai/seed.

(21%) causing the adjusted percent infection of the treated flat to exceed 100 and the wrong isolate was poured on one of the treated flats for 12C from 2015; therefore, these two sets of flats were considered as missing data for data analysis.

Inoculum production for the pathogen sensitivity study was not always successful. Flats of seedlings for soil drenching each greenhouse room were inoculated and planted on different days using the whole seedling immersion method. It was usually impossible to determine if the plants in the second set of flats for the second greenhouse room were sufficiently infected when

it was time to select isolates expected to sporulate well for the first room. Therefore, in the first three rounds of trials, an insufficient quantity of infected seedlings was available from the same pathogen isolate to soil drench the flats in both greenhouse rooms, so another isolate was substituted. This made it difficult to compare isolates by trial and required comparing isolates by greenhouse room.

To test for pathogen sensitivity with an obligate pathogen, an inoculated control needs to be used to ensure that the lack of infected treated plants is because of the fungicide and not the infection process. This is especially important since all the isolates had been frozen for varying amounts of time. All the pre-1997 isolates were frozen between 1990 and 1993 and revived in 2014 and 2016 except for the 1985 isolate which was revived in 1992 and 1993 and then replaced with new sporulated cotyledons. Because 61% of the pre-1997 isolates in the freezer were not viable, a comparison needed to be made between the aggressiveness of the pre-1997 isolates and the 2014 and 2015 isolates. Means for the flats with nontreated seed for both groups of pathogen isolates were not compared using a t-test, because the goal was 100% seedling infection and the data was not normally distributed. Mean percent infection for the pre-1997 isolates was 92%, while mean percent infection for the 2014 and 2015 isolates was 94%, indicating that *P. halstedii* isolates subjected to long-term freezing, were still aggressive enough to reliably infect susceptible seedlings. Of the 26 flats of nontreated seed with less than 70% infection out of 390 flats (7%), 13 flats were inoculated with pre-1997 isolates and 13 flats were inoculated with 2014 and 2015 isolates.

For each trial, the two reference isolates, Enderlin #2 305B from 1992 and 54A from 2015, were supposed to be used for an assay reproducibility calculation used to generate approximate limits for a 95% confidence interval (Wong and Wilcox 2002). This reproducibility

calculation was designed for laboratory leaf disc studies, not the more variable greenhouse environment. A disproportionally large number of flats with nontreated seed with less than 70% infection were from the two reference isolates, 7 of 48 flats (15%). The variability in infection of these reference isolates with or without a suppressive fungicide indicated that environmental conditions favoring downy mildew were lacking in certain locations in the greenhouse rooms depending on the weather outside. Levene's and Brown and Forsythe's test for homogeneity of variance was run separately for these two isolates and both variances exceeded 0.05 indicating homogeneity of variance between greenhouse rooms. The general linear model procedure to conduct an analysis of variance (ANOVA) on the mean percent infection for isolate Enderlin #2 305B from 1992 indicated no significant effect of the two greenhouse rooms. At the 95% level of confidence, there was a significant effect for isolate 54A between the two rooms.

Trials in each greenhouse were analyzed separately using SAS to test for homogeneity of variance using Levene's and Brown and Forsythe's tests. Variances were not homogeneous; therefore, data was transformed in Excel by using the formula =SQRT (adjusted % infection+0.5) to modify the data due to the violation of assumptions of normality and heterogeneity of variance. The 0.5 was added to each adjusted percent infection because a value of zero cannot be transformed using either log or square root transformations. The square root transformation was chosen because it is commonly used when the results are a count of something (McDonald 2014). Trials in each greenhouse were analyzed separately again using Levene's and Brown and Forsythe's tests for homogeneity of variance and using the general linear model procedure to conduct an analysis of variance (ANOVA) on the mean percent infection to determine whether the trials could be combined. In both homogeneity of variance tests the variances were >0.05, so the data from the two greenhouses could be combined. Four

isolates from each treatment group did show a significant effect in mean percent infection between greenhouse rooms.

The hypothesis being tested in this study was a comparison of the treatment means of the pre-1997 and 2014-2015 isolates, not a comparison of the efficacy of the fungicide azoxystrobin in the two inoculated subplots. Therefore, the SAS mixed model procedure for split plot RCBD was inappropriate for this kind of trial. This experiment needed to be a split plot design, to increase precision in estimating the percent infection due to the treated seed as compared to the nontreated seed. After calculating this percent infection, the results from the nontreated portion of the subplot has little value. Additionally, because randomization of a split plot RCBD requires two randomizations, the error term for block effects and variability between replications of an isolate increases. The more appropriate test for the hypothesis was a t-test between two treatment means, pre-1997 isolates and 2014-2015 isolates. The folded f-test for homogeneity of variances showed homogeneity between isolates in the two treatment groups and the pooled method t-test procedure at the 95% level of confidence showed a highly significant difference in sensitivity to azoxystrobin between the pre-1997 and 2014-2015 isolates.

Mean percent infection was 9.6% for pre-1997 isolates and 15% for 2014 and 2015 isolates. The frequency histogram showing mean percent infection of each isolate as a percentage of the total number of isolates in the treatment group indicates a slight shift towards reduced sensitivity to azoxystrobin by some isolates (Figure 3.2). Mean percent infection for all but one of the pre-1997 isolates was below 15%. For the pre-1997 isolates, four isolates were between 0 and 5% infection, six isolates were between 5 and 10% infection, eight isolates were between 10 and 15% infection and one isolate was between 25 and 30% infection. For the 2014 and 2015 isolates, one isolate was between 0 and 5% infection, ten isolates were between 5 and

10% infection, twelve isolates were between 10 and 15% infection, eight isolates were between 15 and 20% infection, six isolates were between 20 and 25% infection and there was one isolate in each of the next three categories of 25 to 30% infection, 30 to 35% infection and 35 to 40% infection.

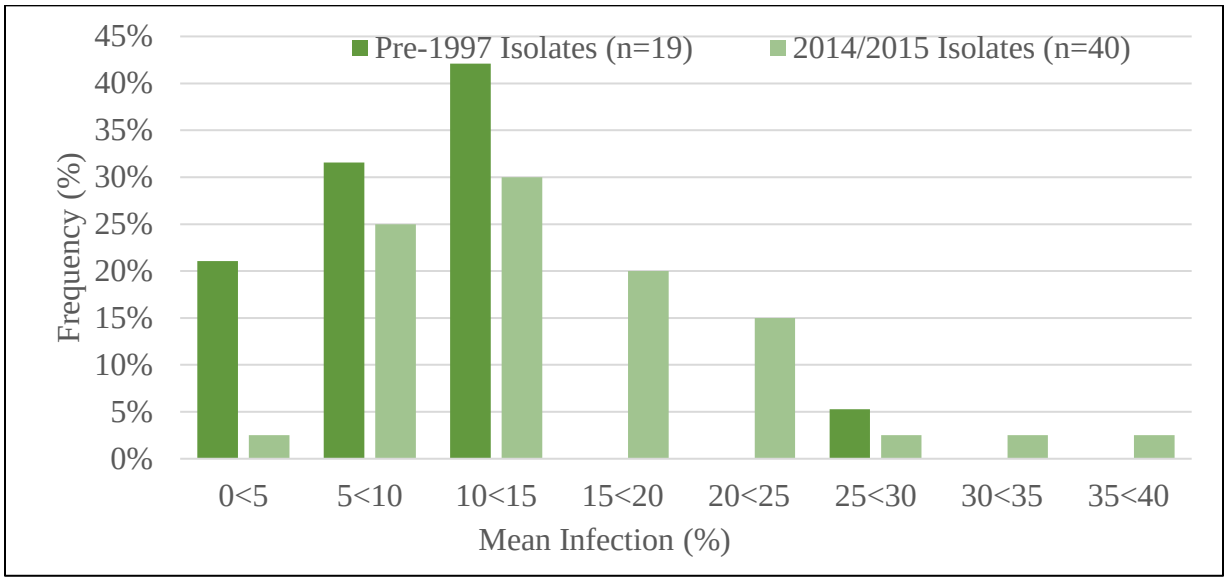

Figure 3.2. Frequency histogram of mean percent infection of pre-1997 and 2014-2015 isolates treated with 10 ug ai/seed of azoxystrobin and inoculated with a spore suspension of *Plasmopara halstedii*

Discussion

Azoxystrobin significantly reduced infection on all 21 *P. halstedii* isolates selected from 1985 to 1994, prior to the use of any fungicidal products containing azoxystrobin, and on all 42 isolates selected from the 2014-2015 survey, after a decade of use of azoxystrobin as a sunflower seed treatment. No isolate approached infection levels found in the inoculated, nontreated controls. As such, the pathogen isolates evaluated are still considered sensitive to azoxystrobin in the greenhouse and azoxystrobin should still suppress downy mildew in the field. However, results of this study also suggest that the 2014 and 2015 isolates have become less sensitive to the chemical compared to pre-1997 isolates. There are multiple explanations that could explain

this shift, only one of which is that the pathogen is developing reduced sensitivity to azoxystrobin.

One possible explanation is that the slight shift could simply be due to variability within the project. The majority of baseline sensitivity and pathogen sensitivity studies have been done on pathogens that are not obligate biotrophs and can be cultured or grown in the lab. This alone reduces variability because of a stable environment in the petri dishes and laboratory. In many cases of fungicide sensitivity experiments on obligate pathogens, a leaf disc method can be used to minimize variability. As an example, leaf disc inoculation is regularly used for in vivo testing of fungicide sensitivity to foliar pathogens such as *P. viticola*. The previous studies using leaf disc inoculation to determine *P. halstedii* sensitivity to metalaxyl were only tested in one laboratory on two resistant isolates, and only to confirm pathogen resistance that had been documented in the field. Because *P. halstedii* is primarily a root pathogen and azxoxystrobin is used as a seed treatment with no confirmed field insensitivity, methodology replicating the natural infection process was considered appropriate for this project. Studies done in growth chambers would definitely have provided more environmental control, but growth chambers large enough to grow 196 flats were not available. As a consequence, all the work was done in a greenhouse environment, dramatically increasing variability over growth chamber studies. Complicating this, *P. halstedii* infects the roots of a living plant, so infection after inoculation is subject to more environmental variables than direct inoculation to a leaf. Similarly, successful infection is only manifested as a systemic response; the hyphae grow up the inside of the plant in order to emerge from stomata and produce visible zoosporangiophores.

One of the hardest variables to control in a greenhouse environment is temperature, and although it was managed well enough to result in a successful experiment, temperature

fluctuations increased variability in this project. Temperature is a critical environmental factor influencing the infection of seedlings with *P. halstedii* and is also the main factor in maintaining soil moisture, another critical factor influencing infection. Additionally, temperature and soil moisture effect the length of time radicles are at the ideal length for infection and how quickly the seedlings emerge from the sand and perlite mix. To minimize the variability caused by solar heating, the greenhouse rooms used in this study were in the same position along parallel and adjacent greenhouse ranges. To manage temperature to the best of our ability, greenhouse rooms were cooled using shade cloth, vents and water cooling systems, and heated through the use of radiators and fans. However, significant fall temperature swings common in North Dakota caused variability in both rooms due to colder temperatures by the vents, fans blowing more on flats in the center of the room causing drying and heating, door effects and non-optimal vent operation due to occasional mechanical failures. These factors may have had some effect on one of the three replicates for an isolate in the room, but are unlikely to have significantly affected the overall difference in means between the two groups of isolates.

Another source of variability was due to the soil drenching method itself, and its extreme sensitivity to moisture. All flats were watered well the evening before soil drenching, but in the morning prior to drenching the flats only a little water was applied. If too much water was applied, then water and possibly spores drained out of the flats, but if not enough water was applied then the flats started to dry out during the five hours it took to soil drench inoculate the 102 or 96 flats. All the flats were lightly watered six hours after inoculation was completed, so the ones inoculated at 8 a.m. were watered at the same time as the ones inoculated at 12:15 p.m. To limit the effect of this variability the isolates were inoculated in the order they appeared in the randomization for each block. The range in concentrations of 20000 to 40000 spores ml^{-1} in 100

ml of amended water should have had no effect on percent infection, but it was definitely not as uniform a quantity of inoculum as transferring a plug of media from the leading edge of the fungal growth onto fungicide-amended media in a petri dish.

Another possible explanation is that the slight shift in sensitivity between the two groups of isolates could simply be due to isolates from pre-1997 being less aggressive after up to 16 years in freezer storage. However, analysis demonstrated that this was not the case. Mean percent infection for the pre-1997 isolates was 92%, while mean percent infection for the 2014 and 2015 isolates was 94% indicating that long-term freezing of *P. halstedii* isolates still viable were still aggressive enough to reliably infect susceptible seedlings. Of the 26 flats of nontreated seed with less than 70% infection out of 390 flats (7%), 13 flats were inoculated with pre-1997 isolates and 13 flats were inoculated with 2014 and 2015 isolates.

The final possibility is that the shift in sensitivity between the isolates collected before the use of azoxystrobin and the isolates collected in 2014 and 2015 is the result of mutations associated with QoI resistance. The related foliar oomycete pathogens, *P. viticola*, *P. cubensis* and *Pythium aphanidermatum* have developed insensitivity to azoxystrobin (FRAC 2012; Genet et al. 2006; Gisi et al. 2000; Ishii et al. 2001). The three mutations in the cytochrome b gene that have been found and that differentially effect pathogen sensitivity are G143A, F129L and G137R. The first resistant population of *P. viticola*, in the same genus as *P. halstedii*, was found in 1997 due to the G143A mutation (Heaney et al. 2000). F129L has also been found in very low percentages in *P. viticola* (Gisi and Sierotzki 2015). *P. cubensis* has the G143A mutation and *P. aphanidermatum* has the F129L mutation (FRAC 2012). Resistance factors were calculated as the mean percent infection of the isolate divided by the mean percent infection of all the isolates collected prior to 1997. The resistance factors for the three *P. halstedii* isolates

that were above the range of the pre-1997 isolates were 2.7, 3.4 and 3.9. This slight shift in no way looks like the complete resistance found with the G143A mutation, with typical resistance factor values in the hundreds. The G137R mutation is unlikely, because it is extremely rare in nature and has only been documented once. A typical sensitivity shift for the F129L mutation varies with the pathogen and the individual QoI fungicide, and has not been clearly defined. In an in vitro study on *Alterniaria solani*, the resistance factor based on mean EC_{50} for isolates with the F129L mutation was 13x for azoxystrobin (Pasche et al. 2004; 2005). Disease control was still effective with other related fungicides for management of *A. solani* with a two- to threefold shift based on mean EC_{50} in in vitro sensitivity (Pasche et al. 2005). A F129L mutation in the three pathogen isolates with mean percent infection above the range of the pre-1997 isolates is possible. The effect of the F129L and the G137R mutations on fungicide sensitivity is considered to be a reduction in sensitivity, not insensitivity, which remains below levels that would cause the fungicide not to be effective in the field.

This study was conducted at the request of the sunflower industry and stakeholders, due to concerns of high incidence in downy mildew occurring in fields treated with azoxystrobin, a fungicide labeled only for suppression. Despite the complexities commensurate with an obligate root-infecting, systemic pathogen, a greenhouse environment, lack of documented field failure and a suppressive fungicide, data strongly suggests two things. First, no isolates tested approached the infection level occurring on the inoculated, nontreated controls, strongly suggesting that *P. halstedii* has not become insensitive to azoxystrobin. Secondly, a slight shift in mean sensitivity between pre-1997 and recently collected isolates, that is difficult to explain, was observed. Taken together, it is our recommendation that future work is prudent. Given the development of the baseline in this study, greenhouse-based monitoring using a range of

concentrations could be done on a case by case basis when a high level of downy mildew occurs in the field. If true insensitivity or reduced sensitivity above the level found in this study is found, it would be prudent to develop PCR primers for the mutations detected, making the identification much easier. Finally, to further analyze the slight shift observed in this greenhouse-based study, a smaller subset of isolates could be tested in a more controlled environment (i.e., growth chamber). This last course of action is thought to be more academic then actionable, since the infection on no isolate approached that of the nontreated control and field failure has not been documented.

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APPENDIX A. INCIDENCE AND PREVALENCE OF DOWNY MILDEW IN NORTH DAKOTA AND NORTHERN SOUTH DAKOTA IN 2014 AND 2015

Table A1. Incidence and prevalence of downy mildew in North Dakota and northern South Dakota in 2014.

		All Fields	North Dakota	South Dakota
Total Fields		104	87	17
Incidence or Occurrence[a] (number of fields)	0	37	31	6
	0, present	31	26	5
	0.5<5%	26	22	4
	5<15%	9	7	2
	15<25%	1	1	0
	25<50%	0	0	0
	50-100%	0	0	0
Prevalence (%)[b]		64.4	64.4	64.7

[a]Incidence was determined for each field surveyed and is defined as the percentage of plants infected out of 200 plants. Occurrence is defined as having observed downy mildew in a field in trace quantities where the disease was present, but was not observed in the 200 plants used to calculate incidence at the survey points.

[b]Prevalence was determined based on whether the disease was present or absent in a field. Downy mildew was considered present if one plant with symptoms was identified anywhere in the field; therefore, a field with zero incidence in the 200 plants assessed was considered infected with downy mildew if a plant was found elsewhere in the field. Prevalence is defined as the percent of fields with downy mildew.

Table A2. Incidence and prevalence of downy mildew in North Dakota and northern South Dakota in 2015.

		All Fields	North Dakota	South Dakota
Total Fields		76	69	7
Incidence or Occurrence[a] (number of fields)	0	17	16	1
	0, present	25	21	4
	0.5<5%	18	17	1
	5<15%	11	10	1
	15<25%	4	4	0
	25<50%	1	1	0
	50-100%	0	0	0
Prevalence (%)[b]		77.6	76.8	85.7

[a]Incidence was determined for each field surveyed and is defined as the percentage of plants infected out of 200 plants. Occurrence is defined as having observed downy mildew in a field in trace quantities where the disease was present, but was not observed in the 200 plants used to calculate incidence at the survey points.

[b]Prevalence was determined based on whether the disease was present or absent in a field. Downy mildew was considered present if one plant with symptoms was identified anywhere in the field; therefore, a field with zero incidence in the 200 plants assessed was considered infected with downy mildew if a plant was found elsewhere in the field. Prevalence is defined as the percent of fields with downy mildew.

Table A3. Incidence and prevalence of downy mildew in North Dakota fields in 2014 and 2015, as determined by this study, the NSDU-IPM survey and the NSA survey based on the same counties in North Dakota.

Surveys	2014[a]	2014 NDSU-IPM[b]	2015[a]	2015 NDSU-IPM[b]	2015 NSA[c]
Number of Fields Surveyed	87	99	69	127	55
% Field Incidence[d] (%)					
0.5<5%	25.3	40.4	24.6	18.9	16.4
5<15%	8.0	15.2	14.5	5.5	5.5
15<25%	1.1	4.0	5.8	3.9	3.6
25<50%	0.0	1.0	1.4	3.1	0.0
50<100%	0.0	0.0	0.0	1.6	0.0
Mean % Incidence Across Fields	1.0	3.1	3.3	2.9	1.4
Prevalence (%)					
Based on downy mildew incidence ≥0.5% at survey points	34.5	60.6	46.4	33.1	25.5
Based on downy mildew present anywhere in field	64.4	-	76.8	-	-

[a]For this study, field incidence was based on 40 plants at five points in an inverted W-shaped pattern for a total of 200 plants.
[b]North Dakota State University-Integrated Pest Management (NDSU-IPM) survey (Knodel 2014; 2015). Field incidence was based on 40 plants at five points in an inverted W-shaped pattern for a total of 200 plants.
[c]National Sunflower Association (NSA) Crop Survey (Kandel and Gulya 2016). Field incidence was based on 25 consecutive plants in two rows at two points in the field for a total of 100 plants.
[d]Incidence was determined for each field surveyed and is defined as the percentage of plants infected out of the number of plants inspected. Percent of fields was calculated based on the number of fields in each category divided by the total number of fields.

APPENDIX B. VIRULENCE PHENOTYPES OF NORTH DAKOTA, SOUTH DAKOTA, MINNESOTA AND NEBRASKA *PLASMOPARA HALSTEDII* ISOLATES ON AN EXPANDED SET OF SUNFLOWER DIFFERENTIALS

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials.

Isolate	Date Rated	Race[a]	Differential Line[b]																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1C, ND	1/27/15	7006_0	SI	SI	SI	RI	RI	RI	RI	RI	RI	RII	SI	SI	RI		RI	RI	RI	RI
2A, ND	12/30/14	700600	SI	SI	SI	RI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	RII
4A, ND	12/30/14	714700	SI	SI	SII	SI	RII	RII	RI	RI	SI	SI	SI	SII	RII	RII	RI	RI	RI	RII
	2/17/15	714700	SI												RII					
	4/18/15	714700	SI								SI	SI	SI	RI	RI	RII				
7C, ND	2/26/15	710	SI	SI	SI	SI	RII	RII	RI	RI	RI						RI		RI	RI
8A, ND	3/2/15	707	SI	SII	SI	RI	RI	RI	SII	SII	SI						RI		RI	
	3/18/15	7077__	SI	SII	SI	RI	RI	RI	SI	SI	SI	SI	SI	SI	SII			RI		
	4/18/15	70771_	SI						SI	SI	SII	SII	SI	SI	SI	RI				
	5/10/17	707710	SI																	RI
9B, ND	3/5/15	700	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI		RI	
10A, ND	12/30/14	710600	SI	SI	SI	SI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RII	RI	RI	RI	RII
11A, ND	1/27/15	7106_0	SI	SI	SI	SI	RII	RII	RI	RI	RI	RII	SI	SI	RI		RI	RI	RI	RI
12A, ND	12/30/14	714710	SI	SI	SI	SI	RII	RII	RI	RI	SI	SI	SI	SI	SI	RII	RI	RI	RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race	Differential Line																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
16B, ND	3/3/15	710	SI	SI	SI	SI	RI	RI	RI	RI	RI						RI		RI	
18B, ND	2/16/15	3143_0	SI	SII	RII	SI	RI	RI	RI	RI	SI	SI	SII	RI			RI	RI	RI	RII
22D, ND	2/11/15	7106_0	SI	SI	SI	SI	RII	RI	RI	RI	RI	RII	SI	SI			RI	RI	RI	RI
25A, ND	2/16/15	7106_0	SI	SI	SI	SI	RII	RI	RI	RI	RI	RII	SI	SI			RI	RI	RI	RI
26A, ND	12/30/14	700600	SI	SI	SI	RI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	RII
27B, ND	2/16/15	7147_0	SI	SII	SI	SI	RII	RI	RI	RI	SI	SI	SI	SI			RI	RI	RI	RI
28C, ND	2/16/15	7106_0	SI	SI	SI	SI	RI	RI	RI	RI	RI	RI	SI	SI			RI	RI	RI	RI
29C, ND	1/27/15	7147_0	SI	SI	SI	SI	RI	RI	RI	RI	SI	SII	SI	SI	SI		RI	RI	RI	RI
30B, ND	2/16/15	7106_0	SI	SI	SI	SI	RI	RI	RI	RI	RI	RI	SI	SI			RI	RI	RI	RI
31A, ND	12/30/14	710600	SI	SI	SII	SI	RI	RI	RI	RI	RI	RII	SI	SI	RI	RII	RI	RI	RI	RII
32C, ND	2/11/15	304300	SI	SI	RII	RI	RI	RI	RI	RI	SI	SI	SI	RII			RI	RI	RI	RI
	2/27/15	304300	SI	SI	RII	RI	RI	RI	RI	RI	SI						RI		RI	
	4/18/15	304300	SI								SI				RII	RI				
33C, ND	1/27/15	7147_0	SI	SII	SI	SI	RII	RI	RI	RI	SI	SI	SI	SI	SI		RI	RI	RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race	Differential Line																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
34B, ND	2/16/15	3143_0	SI	SI	RII	SI	RII	RII	RI	RI	SI	SI	SI	RII			RI	RI	RI	RI
35B, ND	12/30/14	710600	SI	SI	SI	SI	RII	RII	RI	RI	RI	RII	SI	SI	RI	RII	RI	RI	RI	RII
40A, ND	2/11/15	7006_0	SI	SI	SI	RI	RI	RI	RI	RI	RI	RI	SI	SI			RI	RI	RI	RI
43A, ND	1/27/15	7747_0	SI	SI	SI	SI	SI	SI	RI	RI	SI	SI	SI	SI	SI		RI	RI	RI	RI
44A, ND	12/30/14	774730	SI	SI	SI	SI	SI	SI	RI	RI	SI	SI	SI	SI	SI	SI	RI	RI	RI	RII
45A, ND	3/5/15	700	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI		RI	
48A, ND	2/26/15	714	SI	SI	SI	SI	RI	RI	RI	RI	SI						RI		RI	RI
49A, ND	2/26/15	710	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI		RI	-
50A, ND	2/26/15	714	SI	SI	SI	SI	RII	RII	RI	RI	SI						RI		RI	RI
51A, ND	2/11/15	700602	SI	SI	SI	RI	RI	RI	RI	RI	RI	RI	SI	SI			RI	RI	SI	RI
	2/27/15	700602	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI		SII	
	4/18/15	700602	SI									RI	SI	SI	RI	RI	RI		SI	
54A, ND	1/5/15	714__0	SI	SII	SII	SI	RI	RI	RI	RI	SI		SI	SII	SII		RI	RI	RI	RI
55A, SD	2/11/15	7147__	SI	SI	SI	SI	RII	RII	RII	RI	SI	SI	SI	SI			RI	RI	RI	

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race	Differential Line																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
56B, SD	2/16/15	7147_0	SI	SI	SII	SI	RII	RII	RI	RI	SI	SII	SI	SI			RI	RI	RI	RI
57A, SD	2/11/15	710__0															RI	RI	RI	RI
	2/27/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI		RI	
59A, SD	3/5/15	714	SI	SII	SI	SI	RI	RI	RI	RI	SI						RI		RI	
60A, SD	3/5/15	714	SI	SII	SI	SI	RI	RI	RI	RI	SI						RI		RI	
62A, SD	1/27/15	7747_0	SI	SI	SI	SI	SI	SI	RI	RI	SI	SII	SI	SI	SI		RI	RI	RI	RI
63A, SD	2/16/15	7106_0	SI	SI	SI	SI	RI	RI	RI	RI	RI	RI	SI	SI			RI	RI	RI	RI
64A, SD	2/16/15	7747_0	SI	SII	SI	SI	SI	SII	RI	RI	SI	SI	SI	SI			RI	RI	RI	RI
65A, SD	3/5/15	700	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI		RI	
67A, SD	1/27/15	7747_0	SI	SI	SI	SI	SI	SII	RI	RI	SI	SI	SI	SI	SI		RI	RI	RI	RI
71A, SD	12/23/14	71060_	SI	SI	SI	SI	RI	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	
71B, SD	12/23/14	71060_	SI	SI	SI	SI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	
71C, SD	12/23/14	71060_	SI	SI	SI	SI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RII	RI	RI	RI	
71D, SD	12/23/14	710600	SI	SI	SI	SI	RII	RI	RI	RI	RI	RII	SI	SI	RI	RII	RI	RI	RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race	Differential Line																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
71E, SD	12/23/14	710600	SI	SI	SI	SI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RII	RI	RI	RI	RII
71F, SD	12/23/14	710600	SI	SI	SI	SI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RII	RI	RI	RI	RII
72B, ND	1/27/15	7006_0	SI	SI	SI	RI	RI	RI	RI	RI	RI	RI	SI	SI	RI		RI	RI	RI	RI
73A, ND	1/5/15	7106_0	SI	SII	SII	SI	RII	RI	RI	RI	RI	RI	SI	SI	RI		RI	RI	RI	RI
74B, ND	2/26/15	774	SI	SI	SI	SI	SI	SI	RI	RI	SI						RI		RI	-
77A, ND	12/30/14	714710	SI	SI	SI	SI	RII	RI	RI	RI	SI	SI	SI	SI	SI	RII	RI	RI	RI	RII
81A, ND	1/27/15	7747_0	SI	SI	SI	SI	SI	SI	RI	RI	SI	SI	SI	SI	SI		RI	RI	RI	RI
82C, ND	2/26/15	714	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI		RI	RI
83A, ND	2/26/15	774	SI	SI	SI	SI	SI	SII	RI	RI	SI						RI		RI	RI
84A, ND	1/5/15	7106__	SI	SI	SI	SI	RI	RI	RI	RI	RI	RI	SI	SII	RI		RI	RI	RI	
85A, ND	2/26/15	710	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI		RI	RI
88A, ND	2/16/15	7106_0	SI	SI	SII	SI	RII	RI	RI	RI	RI	RI	SI	SI			RI	RI	RI	RI
89B, ND	12/30/14	710600	SI	SI	SI	SI	RI	RI	RI	RI	RI	RII	SI	SII	RI	RII	RI	RI	RI	RII

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race	Differential Line																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
91A, ND	3/3/15	71060_	SI	SI	SII	SI	RI	RI	RI	RI	RI	RII	SI	SI	RI	RII	RI		SI	
	4/18/15	71060_	SI									RII	SI	SI	RI	RII	RI	RI	SI	
	5/10/17	710602	SI																	RI
92A, ND	1/5/15	7747_0	SI	SII	SI	SI	SI	SII	RI	RI	SI	SI	SI	SI	SI		RI	RI	RI	RI
93B, ND	12/30/14	714710	SI	SII	SI	SI	RII	RI	RI	RI	SI	SI	SI	SI	SI	RII	RII	RI	RI	RI
94C, ND	12/30/14	710600	SI	SI	SI	SI	RII	RII	RI	RI	RI	RII	SI	SI	RI	RII	RI	RI	RI	RI
96A, ND	3/5/15	714	SI	SI	SI	SI	RI	RI	RI	RI	SI						RI		RI	
97B, ND	2/16/15	7106_0	SI	SI	SI	SI	RII	RII	RI	RI	RI	RI	SII	SI			RI	RI	RI	RI
98B, ND	3/5/15	700	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI		RI	
99C, ND	3/3/15	714	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI		RI	
100B, ND	2/23/15	714	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI		RI	
101A, ND	2/26/15	710	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI		RI	RI
102A, ND	12/30/14	710600	SI	SI	SI	SI	RII	RI	RI	RI	RI	RII	SI	SI	RI	RI	RI	RI	RI	RII
103A, ND	2/26/15	700	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI		RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race	Differential Line																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
105E, ND	12/30/14	700600	SI	SI	SI	RI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	RII
Fargo, ND	12/30/14	710600	SI	SI	SI	SI	RII	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	RII
Grace City B, ND	12/23/14	70060_	SI	SI	SI	RI	RI	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	
Grandin, ND	1/27/15	7347__	SI	SI	SI	SI	SII	RII	RI	RI	SI	SI	SI	SI	SII		RI	RI	RI	
	5/16/15	73471_	SI													RI				
	5/10/17	734710	SI																	RI
Linton A, ND	12/23/14	71471_	SI	SI	SI	SI	RI	RI	RI	RI	SII	SI	SI	SI	SI	RI	RI	RI	RI	
Linton B, ND	12/23/14	714710	SI	SII	SI	SI	RII	RI	RI	RI	SI	SI	SI	SI	SI	RI	RI	RI	RI	RI
Linton C, ND	12/23/14	714710	SI	SII	SI	SI	RI	RI	RI	RI	SI	SI	SI	SI	SI	RII	RI	RI	RI	RI
Mandan, ND	12/23/14	70471_	SI	SII	SI	RI	RI	RI	RI	RI	SI	SI	SI	SI	SI	RI	RI	RI	RI	
	5/10/17	704710	SI																	RI
Agar, SD	2/10/15	7147__	SI	SI	SI	SI	RII	RII	RI	RI	SI	SI	SI	SI			RI	RI	RII	
Onida, SD	12/23/14	70060_	SI	SI	SI	RI	RI	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race	Differential Line																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Opsahl NW, MN	12/23/14	70060_	SI	SI	SI	RI	RII	RI	RI	RI	RI	RI	SII	SI	RI	RI	RI	RI	RI	
Opsahl SE, MN	12/23/14	700600	SI	SI	SI	RI	RI	RI	RI	RI	RI	RII	SI	SI	RI	RI	RI	RI	RI	RI
Opsahl SW, MN	12/23/14	710600	SI	SI	SI	SI	RII	RI	RI	RI	RI	RII	SI	SI	RI	RI	RI	RI	RI	RII
Rothsay, MN	2/10/15	7106_0	SI	SI	SI	SI	RII	RII	RI	RI	RI	RI	SI	SI			RI	RI	RI	RI
Staples, MN	1/27/15	7006_0	SI	SI	SI	RI	RI	RI	RI	RI	RI	RII	SI	SI	RI		RI	RI	RI	RI
26 13-21, MN	12/30/14	700	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI		RI	
26 17-49, MN	12/30/14	714	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI		RI	
26 18-57, MN	12/30/14	714	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI		RI	
26 18-61, MN	12/30/14	714	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI		RI	
26 5-29, MN	12/30/14	714	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI		RI	
26 8-53, MN	12/30/14	710	SI	SI	SII	SI	RII	RII	RI	RI	RI						RI		RI	
26 9-29, MN	12/30/14	700	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI		RI	
29 11-13, MN	12/30/14	714	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI		RI	

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B1. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race	Differential Line																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
29 11-49, MN	12/30/14	714	SI	SII	SI	SI	RI	RI	RI	RI	SI						RI		RI	
29 12-29, MN	12/30/14	714	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI		RI	
29 12-33, MN	12/30/14	714	SI	SII	SI	SI	RII	RI	RI	RI	SI						RII		RI	
29 13-13, MN	12/30/14	714	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI		RI	
29 13-53, MN	12/30/14	714	SI	SII	SII	SI	RI	RI	RI	RI	SI						RI		RI	
29 2-25, MN	12/30/14	714	SI	SII	SI	SI	RII	RI	RI	RI	SI						RII		RII	
29 2-33, MN	12/30/14	714	SI	SI	SI	SI	RII	RI	RII	RI	SI						RI		RI	
29 2-5, MN	12/30/14	714	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI		RI	
29 2-61, MN	12/30/14	714	SI	SII	SII	SI	RII	RI	RI	RI	SI						RI		RI	
29 2-65, MN	12/30/14	714	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI		RI	

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials.

Isolate	Date Rated	Race[a]	Differential Line[b]																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1C, ND	11/25/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI
3C, ND	12/15/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RII						RI	RI	RII	RI
4A, ND	12/15/15	710__0	SI	SII	SI	SI	RII	RI	RI	RI	RII						RI	RI	RII	RI
5C, ND	11/25/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	RI
6C, ND	12/11/15	710__0	SI	SI	SII	SI	RI	RI	RI	RI	RI						RI	RI	RI	RI
7B, ND	12/15/15	714__0	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI	RI	RII	RI
8B, ND	11/24/15	714__0	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI	RI	RI	RI
9C, ND	12/11/15	700__0	SI	SI	SII	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI
10A, ND	12/16/15	314__0	SI	SI	RII	SI	RII	RII	RI	RI	SI						RI	RI	RI	RI
11B, ND	12/28/15	314300	SI									SI	SI	RI	RI	RI				
	12/15-16/15	314__0	SI	SI	RII	SI	RI	RI	RI	RI	SI						RI	RI	RI	RI
12C, ND	11/25/15	700__0	SI	SI	SI	RI	RI	RII	RI	RI	RII						RI	RI	RII	RI
13A, ND	12/16/15	714__0	SI	SI	SII	SII	RII	RII	RI	RI	SI						RI	RI	RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

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Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
14B, ND	12/16/15	704__0	SI	SI	SI	RI	RI	RI	RI	RI	SI						RI	RI	RI	RI
15B, ND	12/16/15	714__0	SI	SI	SI	SI	RII	RII	RI	RI	SI						RI	RI	RI	RI
16C, ND	12/22/15	704__0	SI	SII	SI	RI	RI	RI	RI	RI	SI						RI	RI	RI	RI
	1/5/16	704710	SI									SI	SI	SI	SI	RI				
17C, ND	12/22/15	710__2	SI	SI	SI	SI	RII	RII	RI	RI	RII						RI	RI	SI	RI
	1/7/16	710602	SI									RI	SI	SI	RI	RI			SII	
18B, ND	12/16/15	704__0	SI	SI	SI	RI	RI	RI	RI	RI	SII						RI	RI	RII	RI
20A, ND	12/22/15	717__0	SI	SII	SI	SI	RII	RII	SI	SII	SI						RI	RI	RII	RI
	1/5/16	717710	SI						SI	SII	SI	SI	SI	SI	SI	RII				
21B, ND	12/22/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	RI
22B, ND	12/22/15	704__0	SI	SII	SI	RI	RI	RI	RI	RI	SI						RI	RI	RII	RI
	1/5/16	704710	SI									SI	SI	SI	SI	RI				

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
23A, ND	12/8/15	314__0	SI	SI	RII	SI	RII	RI	RI	RI	SI						RI	RI	RI	RI
	12/22/15		SI									SI	SI	SI	RII	RII				
	1/5/16		SI											RI						
	2/17/16	314300	SI									SI	SI	RI						
25A, ND	12/22/15	710__2	SI	SI	SI	SI	RI	RI	RI	RI	RI						RI	RI	SI	RI
	1/7/16	710602	SI									RI	SI	SI	RI	RI			SII	
26A, ND	12/22/15	700__2	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	SI	RI
	1/7/16	700602	SI									RI	SI	SI	RI	RI			SI	
27A, ND	12/22/15	710__2	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	SI	RI
	1/7/16	710702	SI									SI	SI	SI	RI	RI			SI	
	2/17/16	710702	SI									SI	SI	SI						
	3/16/16	710702	SI								RI	SI							SI	

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

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Isolate	Date Rated	Race[a]	Differential Line[b]																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
28C, ND	11/25/15	314__0	SI	SI	RII	SI	RII	RI	RI	RI	SI						RI	RI	RII	RI
	12/15/15	314300	SI		RII			RII				SI	SI	RII	RII	RII				
29A, ND	11/25/15	774__0	SI	SI	SII	SI	SI	SI	RI	RI	SI						RI	RI	RI	RI
	3/16/16	774730	SI								SI	SI	SI	SI	SI	SI				
30D, ND	12/28/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	RI
31A, ND	1/7/16	710600	SI	SI	SI	SI	RI	RI	RI	RI	RI	RI	SI	SI	RI	RI	RI	RI	RI	RI
32A, ND	12/28/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI
33B, ND	11/24/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	RI
34C, ND	11/24/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RII	RI
35A, ND	12/28/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RII	RI
36C, ND	12/8/15	714__0	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI	RI	RI	RI
37B, ND	12/16/15	714__0	SI	SI	SI	SII	RII	RII	RII	RI	SI						RI	RI	RII	RI
39A, ND	12/28/15	710__0	SI	SI	SI	SI	RII	RII	RI	RI	RI						RI	RI	RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
40B, ND	11/17/15	770__0	SI	SI	SI	SI	SI	SI	RI	RI	RI						RI	RI	RI	RI
	12/28/15	770620	SI									RI	SI	SI	RI	SII				
	1/7/16	770620	SI	SI	SII	SI	SI	SI				RI	SI	SI	RI	SI				
42B, ND	11/17/15	714__0	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI	RI	RI	RI
45A, SD	12/28/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI
46A, SD	12/28/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI
47A, SD	12/28/15	710__0	SI	SI	SI	SI	RII	RII	RI	RI	RI						RI	RI	RI	RI
48A, SD	12/28/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RII	RI
49A, SD	12/28/15	710__0	SI	SI	SI	SI	RII	RII	RI	RI	RI						RI	RI	RI	RI
50A, ND	11/17/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI
51B, ND	11/17/15	710__2	SI	SI	SI	SI	RI	RI	RI	RI	RI						RI	RI	SI	RI
	3/16/16	710602	SI								RI	RI	SI	SI	RI	RI			SI	
54A, ND	11/17/15	714__0	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI	RI	RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
56A, ND	11/17/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	RI
57A, ND	11/20/15	710__0	SI	SI	SI	SI	RII	RII	RI	RI	RI						RI	RI	RI	RI
	12/10/15		SI				RII	RII				RI		SI		RII				
	12/28/15	710600	SI									RI	SI		RI					
58A, ND	11/20/15	774__0	SI	SI	SI	SI	SI	SI	RI	RI	SI						RI	RI	RII	RI
	3/16/16	774730	SI								SI	SI	SI	SI	SI	SI				
59D, ND	11/20/15	714__0	SI	SII	SI	SI	RII	RI	RI	RI	SI						RI	RI	RII	RI
	12/10/15	714710	SI				RII					SI	SI	SI	SI	RI				
60C, ND	11/20/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RII	RI
61D, ND	11/24/15	710__0	SI	SI	SI	SI	RII	RII	RI	RI	RI						RI	RI	RI	RI
	12/15/15	710600	SI									RI	SI	SI	RI	RII				
62A, ND	11/24/15	714__0	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI	RI	RII	RI
63B, ND	11/24/15	714__0	SI	SI	SI	SI	RII	RII	RI	RI	SI						RI	RI	RII	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
64A, ND	11/24/15	714__0	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI	RI	RII	RI
64A, ND	12/10/15	714710	SI				RII					SI	SI	SI	SI	RII				
67A, ND	12/8/15	314__0	SI	SI	RII	SI	RII	RII	RI	RI	SI						RI	RI	RI	RI
	12/23/15		SI									SI	SI	SI	RI	RII				
	1/5/16		SI											RI						
	2/17/16	314300	SI									SI	SI	RI						
68A, ND	12/16/15	714__0	SI	SI	SII	SI	RII	RII	RI	RI	SI						RI	RI	RII	RI
71C, ND	12/16/15	700__0	SI	SI	SII	RI	RI	RI	RI	RI	RI						RI	RI	RII	RI
72A, ND	12/16/15	714__0	SI	SI	SI	SII	RII	RII	RI	RI	SI						RI	RI	RII	RI
Carrington A, ND	12/1/15	714__0	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI	RI	RII	RI
	12/1/15	314__0	SI	SI	RII	SI	RII	RII	RI	RI	SI						RI	RI	RI	RI
Casselton C, ND	12/15/15	314300	SI		RII		RII	RII				SI	SI	RII	RI	RII				

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Colfax C, ND	11/10/15	714	SI	SII	SI	SI	RII	RII	RI	RI	SI						RI	RI	RI	
	11/30/15	714__0	SI																	RI
Grand Forks B, ND	11/10/15	314	SI	SI	RII	SI	RII	RI	RI	RI	SI						RI	RI	RI	RI
	12/10/15		SI		RII		RII					SI		RI		RII				
	12/28/15	314300	SI									SI	SI		RI					
	1/7/16	314300	SI									SI	SI	RI	RI	RI				
Grandin C, ND	12/1/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	RI
Langdon A, ND	11/10/15	314__0	SI	SI	RII	SI	RII	RII	RI	RI	SI						RI	RI	RI	RI
	12/10/15		SI		RII		RII					SI		RI		RI				
	12/28/15	314300	SI									SI	SI		RI					

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Brookings, SD	11/10/15	314	SI	SI	RII	SI	RII	RI	RI	RI	SI						RI	RI	RI	
	12/10/15		SI		RII		RII					SI		RI		RII				
	12/28/15	31430_	SI									SI	SI		RI					
3-5 Hughes County, SD	12/1/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	RI
4-3 Hughes County, SD	12/1/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RII	RI
5-7 Hughes County, SD	12/1/15	730__0	SI	SI	SI	SI	SII	RII	RI	RI	RI						RI	RI	RI	RI
	12/15/15	730600	SI				SII	RI				RII	SI	SI	RII	RII				
Potter County, SD	11/10/15	714	SI	SI	SI	SI	RII	RI	RI	RI	SI						RI	RI	RI	
	11/30/15	714__0	SI																	RI
1-2 Sully County, SD	12/1/15	700__0	SI	SI	SII	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2-9 Sully County, SD	12/1/15	710__0	SI	SI	SI	SI	RII	RII	RI	RI	RI						RI	RI	RI	RI
Felton B, MN	12/1/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI
Glyndon A, MN	12/1/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI
Hazel A, MN	12/1/15	710__0	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	RI
Rothsay C, MN	12/1/15	314__0	SI	SI	RII	SI	RII	RII	RI	RI	SI						RI	RI	RI	RI
	12/15/15	314300	SI		RII		RII	RII				SI	SI	RII	RII	RII				
Staples C, MN	11/25/15	700__0	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B2. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska on an expanded set of sunflower differentials (continued).

Isolate	Date Rated	Race[a]	Differential Line[b]																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
82 Jefferson C, NE	11/10/15	710	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	
	11/30/15	710__0	SI																	RI
CR 82 Filmore/ Franklin A, NE	11/10/15	710	SI	SI	SI	SI	RII	RI	RI	RI	RI						RI	RI	RI	
	11/30/15	710__0	SI																	RI
Hwy 30 East Side A, NE	11/10/15	700	SI	SI	SII	RI	RI	RI	RI	RI	RI						RI	RI	RI	
	11/30/15	700__0	SI																	RI
Pumpjack E, NE	11/10/15	700	SI	SI	SI	RI	RI	RI	RI	RI	RI						RI	RI	RI	
	11/30/15	700__0	SI																	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.

[b]Internationally accepted differential set of sunflower lines used for determination of virulence phenotype (race) of *P. halstedii* (Gulya et al. 1998; Tourvieille de Labrouhe et al. 2000) with accepted sunflower line substitutions (T. Gulya, *personal communication*); where D-1 is Mycogen 270, D-2 is RHA 265, D-3 is RHA 274, D-4 is DM-2, D-5 is PM 17, D-6 is 803-1, D-7 is HA-R4, D-8 is HA-R5 and D-9 is HA 335. Differential set of sunflower lines proposed to distinguish additional virulence not represented by resistance genes in the original lines (Tourvieille de Labrouhe et al. 2012); where D-10 is Y7Q, D-11 is PSC8, D-12 is XA, D-13 is PSS2RM, D-14 is VAQ and D-15 is RHA 419. Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in the Standard and INRA proposed differential lines; where D-16 is RNID, D-17 is RHA 340 and D-18 is HA 458. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.

Table B3. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska and four supplemental sunflower lines.

Isolate	Race[a]	HA DM 1[b]	RHA 468[b]	TX 16R[b,c]	RHA 428[b]
1C, ND	7006_0	RI	RI	RI (2)	SI
2A, ND	700600	RI	RII	RI (2)	SI
4A, ND	714700	RI	RI	RI (3)	RII
7C, ND	710	RI			
8A, ND	707710	RI	RI	RI	SII
9B, ND	700	RI			
10A, ND	710600	RI	RII	RI (2)	RI
11A, ND	7106_0	RI	RII	RI (3)	SI
12A, ND	714710	RI	RI	RI (1)	SI
16B, ND	710	RI			
18B, ND	3143_0	RI	RI	RI (3)	RI
21C, ND	3143_0	RI	RI	RI (2)	RI
22D, ND	7106_0	RI	RI	RI (3)	SI
25A, ND	7106_0	RI	RI	RI (2)	RI
26A, ND	700600	RI	RI	RI (1)	RI
27B, ND	7147_0	RI	RI	RI (2)	RII
28C, ND	7106_0	RI	RII	RI (1)	RI
29C, ND	7147_0	RI	RI	RI (2)	RI
30B, ND	7106_0	RI	RI	RI	SI
31A, ND	710600	RI	RII	RI (3)	RI
32C, ND	304300	RI	RI	RI (1)	RII
33C, ND	7147_0	RI	RII	RI (4)	RI
34B, ND	3143_0	RI	RI	RI (3)	RI
35B, ND	710600	RI	RII	RI (3)	RI
40A, ND	7006_0	RI	RI	RI (1)	SI
43A, ND	7747_0	RI	RI	RI (3)	RI
44A, ND	774730	RI	RI	RI (4)	SI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.
[b]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in expanded set of differential lines. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.
[c]Number of plants in parentheses signifies the number of plants with atypical susceptible reaction out of 20 plants.

Table B3. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska and four supplemental sunflower lines (continued).

Isolate	Race[a]	HA DM 1[b]	RHA 468[b]	TX 16R[b,c]	RHA 428[b]
45A, ND	700	RI			
48A, ND	714	RI			
50A, ND	714	RI			
51A, ND	700602	RI	RI	RI (2)	RI
54A, ND	714__0	RI	RI	RI (1)	RII
55A, SD	7147__	RI	RI	RI (1)	SI
56B, SD	7147_0	RI	RI	RI (2)	RI
57A, SD	710__0	RI	RI	RI	RI
59A, SD	714	RI			
60A, SD	714	RI			
62A, SD	7747_0	RI	RI	RI (1)	RI
63A, SD	7106_0	RI	RI	RI (6)	SI
64A, SD	7747_0	RI	RI	RI (3)	RI
65A, SD	700	RI			
67A, SD	7747_0	RI	RI	RI (4)	SII
71A, SD	71060_	RI	RI	RI (1)	RI
71B, SD	71060_	RI	RII	RI (1)	RII
71C, SD	71060_	RI	RI	RI (2)	RI
71D, SD	710600	RI	RII	RI (3)	RI
71E, SD	710600	RI	RII	RI (2)	RI
71F, SD	710600	RI	RII	RI (4)	RI
72B, ND	7006_0	RI	RI	RI (4)	SI
73A, ND	7106_0	RI	RI	RI(1)	RI
74B, ND	774	RI			
77A, ND	714710	RII	RII	RI (4)	SI
81A, ND	7747_0	RI	RI	RI (5)	RII

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.
[b]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in expanded set of differential lines. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.
[c]Number of plants in parentheses signifies the number of plants with atypical susceptible reaction out of 20 plants.

Table B3. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska and four supplemental sunflower lines (continued).

Isolate	Race[a]	HA DM 1[b]	RHA 468[b]	TX 16R[b,c]	RHA 428[b]
82C, ND	714	RI			
83A, ND	774	RI			
84A, ND	7106__	RI	RI	RI	RI
88A, ND	7106_0	RI	RI	RI (1)	RI
89B, ND	710600	RI	RI	RI (3)	RI
91A, ND	710602	RI	RI	RI (1)	SI
92A, ND	7747_0	RI	RI	RI (1)	RI
93B, ND	714710	RI	RII	RI (3)	RI
94C, ND	710600	RI	RII	RI (4)	RII
96A, ND	714	RI			
97B, ND	7106_0	RI	RI	RI (1)	RI
98B, ND	700	RI			
99C, ND	714	RI			
100B, ND	714	RI			
101A, ND	710	RI			
102A, ND	710600	RI	RI	RI	RI
103A, ND	700	RI			
105E, ND	700600	RI	RII	RI	RI
Fargo, ND	710600	RI	RI	RI (2)	RI
Grace City B, ND	70060_	RI	RII	RI (1)	RI
Grandin, ND	734710	RI	RII	RI (5)	RI
Linton A, ND	71471_	RI	RII	RI (2)	RII
Linton B, ND	714710	RI	RII	RI (1)	RII
Linton C, ND	714710	RI	RII	RI (2)	RI
Mandan, ND	704710	RI	RI	RI (2)	RI
Agar, SD	7147__	RI	RII	RI	RI
Onida, SD	70060_	RI	RI	RI (3)	RI

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.
[b]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in expanded set of differential lines. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.
[c]Number of plants in parentheses signifies the number of plants with atypical susceptible reaction out of 20 plants.

Table B3. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2014 in North Dakota, South Dakota, Minnesota and Nebraska and four supplemental sunflower lines (continued).

Isolate	Race[a]	HA DM 1[b]	RHA 468[b]	TX 16R[b,c]	RHA 428[b]
Opsahl NW, MN	70060_	RI	RI	RI (1)	RI
Opsahl SE, MN	700600	RI	RII	RI (2)	RI
Opsahl SW, MN	710600	RI	RII	RI (4)	RI
Rothsay, MN	7106_0	RI	RII	RI (2)	RI
Staples, MN	7006_0	RI	RII	RI (3)	SII
26 13-21, MN	700			RI (2)	
26 17-49, MN	714			RI	
26 18-57, MN	714			RI	
26 18-61, MN	714			RI	
26 5-29, MN	714			RI	
26 8-53, MN	710			RI (1)	
26 9-29, MN	700			RI (1)	
29 11-13, MN	714			RI (2)	
29 11-49, MN	714			RI	
29 12-29, MN	714			RI (3)	
29 12-33, MN	714			RI	
29 13-13, MN	714			RI (1)	
29 13-53, MN	714			RI (3)	
29 2-25, MN	714			RI	
29 2-33, MN	714			RI (5)	
29 2-5, MN	714			RI (3)	
29 2-61, MN	714			RI (2)	
29 2-65, MN	714			RI (4)	

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.
[b]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in expanded set of differential lines. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.
[c]Number of plants in parentheses signifies the number of plants with atypical susceptible reaction out of 20 plants.

Table B4. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska and three supplemental sunflower lines.

Isolate	Race[a]	HA DM 1[b]	RHA 468[b]	TX 16R[b,c]
1C, ND	700__0	RI	RI	RI (1)
3C, ND	700__0	RI	RII	RI (4)
4A, ND	710__0	RI	RI	RI (2)
5C, ND	710__0	RI	RI	RI (3)
6C, ND	710__0	RI	RI	RI (3)
7B, ND	714__0	RI	RI	RI (4)
8B, ND	714__0	RI	RI	RI (2)
9C, ND	700__0	RI	RI	RI (1)
10A, ND	314__0	RI	RI	RI (2)
11B, ND	314300	RI	RI	RI (2)
12C, ND	700__0	RI	RI	RI (2)
13A, ND	714__0	RI	RI	RI (1)
14B, ND	704__0	RI	RI	RI
15B, ND	714__0	RI	RII	RI (3)
16C, ND	704710	RI	RI	RI (1)
17C, ND	710602	RI	RI	RI (3)
18B, ND	704__0	RI	RI	RI
20A, ND	717710	RI	RI	RI (1)
21B, ND	710__0	RI	RI	RI (1)
22B, ND	704710	RI	RII	RI (2)
23A, ND	314300	RI	RI	RI (3)
25A, ND	710602	RI	RI	RI
26A, ND	700602	RI	RI	RI (4)
27A, ND	710702	RI	RI	RI (4)
28C, ND	314300	RI	RI	RI (3)
29A, ND	774730	RI	RI	RI (2)
30D, ND	710__0	RI	RII	RI (1)
31A, ND	710600	RI	RI	RI (2)

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.
[b]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in expanded set of differential lines. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.
[c]Number of plants in parentheses signifies the number of plants with atypical susceptible reaction out of 20 plants.

Table B4. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska and three supplemental sunflower lines (continued).

Isolate	Race[a]	HA DM 1[b]	RHA 468[b]	TX 16R[b,c]
32A, ND	700__0	RI	RI	RI (2)
33B, ND	710__0	RI	RII	RI (3)
34C, ND	700__0	RI	RI	RI (2)
35A, ND	710__0	RI	RII	RI (1)
36C, ND	714__0	RI	RI	RI
37B, ND	714__0	RI	RII	RI (3)
39A, ND	710__0	RI	RI	RI (3)
40B, ND	770620	RI	RI	RI
42B, ND	714__0	RI	RI	RI (3)
45A, SD	700__0	RI	RI	RI (2)
46A, SD	700__0	RI	RI	RI (3)
47A, SD	710__0	RI	RI	RI (4)
48A, SD	710__0	RI	RI	RI (2)
49A, SD	710__0	RI	RI	RI (2)
50A, ND	700__0	RI	RII	RI (3)
51B, ND	710602	RI	RII	RI (3)
54A, ND	714__0	RI	RI	RI (4)
56A, ND	710__0	RI	RII	RI (4)
57A, ND	710600	RI	RII	RI (1)
58A, ND	774730	RI	RII	RI (2)
59D, ND	714710	RI	RII	RI (1)
60C, ND	700__0	RI	RII	RI (2)
61D, ND	710600	RI	RII	RI (1)
62A, ND	714__0	RI	RII	RI (3)
63B, ND	714__0	RI	RI	RI (4)
64A, ND	714710	RI	RI	RI (2)
67A, ND	314300	RI	RI	RI (1)

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.
[b]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in expanded set of differential lines. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.
[c]Number of plants in parentheses signifies the number of plants with atypical susceptible reaction out of 20 plants.

Table B4. Virulence phenotypes of *Plasmopara halstedii* isolates collected in 2015 in North Dakota, South Dakota, Minnesota and Nebraska and three supplemental sunflower lines (continued).

Isolate	Race[a]	HA DM 1[b]	RHA 468[b]	TX 16R[b,c]
68A, ND	714__0	RI	RI	RI (4)
71C, ND	700__0	RI	RI	RI (2)
72A, ND	714__0	RI	RII	RI (5)
Carrington A, ND	714__0	RI	RII	RI
Casselton C, ND	314300	RI	RI	RI
Colfax C, ND	714__0	RI	RI	RI (2)
Grand Forks B, ND	314300	RI	RI	RI (2)
Grandin C, ND	710__0	RI	RI	RI
Langdon A, ND	314300	RI	RI	RI (1)
Brookings, SD	31430_	RI	RI	RI (4)
3-5 Hughes County, SD	710__0	RI	RII	RI (1)
4-3 Hughes County, SD	700__0	RI	RI	RI (1)
5-7 Hughes County, SD	730600	RI	RII	RI (5)
Potter County, SD	714__0	RI	RI	RI (3)
1-2 Sully County, SD	700__0	RI	RI	RI (3)
2-9 Sully County, SD	710__0	RI	RII	RI (2)
Felton B, MN	700__0	RI	RII	RI (1)
Glyndon A, MN	700__0	RI	RI	RI (1)
Hazel A, MN	710__0	RI	RI	RI
Rothsay C, MN	314300	RI	RI	RI (2)
Staples C, MN	700__0	RI	RI	RI (2)
82 Jefferson C, NE	710__0	RI	RI	RI
CR 82 Filmore/ Franklin A, NE	710__0	RI	RI	RI (3)
Hwy 30 East Side A, NE	700__0	RI	RI	RI (3)
Pumpjack E, NE	700__0	RI	RI	RI (1)

[a]Race nomenclature according to Gulya et al. (1998) and Tourvieille de Labrouhe et al. (2000), where the first differential line in a set of three is given a 1, the second line a 2, the third line a 4 and then the values for all three lines in the set are added and presented as a single number.
[b]Supplemental sunflower lines used to distinguish additional virulence not represented by resistance genes in expanded set of differential lines. Reactions observed on seedlings 11 to 14 days' post-inoculation using a scale according to Gulya et al. (1998), where scores of RI and RII are considered resistant and scores of SI and SII are considered susceptible. RI indicates resistant, no sporulation, RII indicates weak sporulation on cotyledons, SI indicates susceptible, sporulation on leaves and SII indicates intense sporulation on cotyledons only.
[c]Number of plants in parentheses signifies the number of plants with atypical susceptible reaction out of 20 plants.

APPENDIX C. PATHOGEN SENSITIVITY MATERIAL

Table C1. Mean percent infection by *Plasmopara halstedii* isolate of inoculated, nontreated control seed and azoxystrobin treated seed at 10 ug ai/seed and significance of effect in mean percent infection between greenhouse rooms (<0.05 when comparing rooms).

Isolates	Mean % Infection		Effect Between Greenhouse Rooms
	0 ug ai/seed	10 ug ai/seed	
1985 Clearwater 1357, MN	86.0	1.8	0.6929
1989 Crookston #1657, MN	97.8	7.5	0.0151
1990 Chaffee #1955, ND	96.9	13.8	0.0197
1991 Cass County #2081, ND	96.1	8.9	0.0782
1991 Crookston #19 #2099, MN	98.8	6.2	0.6474
1991 Lisbon 4-111 #1822, ND	86.3	4.7	0.2125
1991 Seedtec Nursery, MN	79.3	5.9	0.0800
1992 Enderlin #1 1C #2293, ND	100.0	13.4	0.2150
1992 Enderlin #2 102A, ND	96.2	15.0	0.0367
1992 Enderlin #2 201C, ND	96.9	27.0	0.5669
1992 Enderlin #2 305B, ND	89.9	8.8	0.0887
1992 Kansas #2261	98.1	11.4	0.2408
1993 Grandin 192 Green, ND	86.4	10.0	0.5099
1993 Grandin 213 Red, ND	94.9	8.1	0.2848
1993 Grandin 381 Red, ND	100.0	3.1	0.0789
1993 Grandin Spray Trial #3, ND	83.8	13.5	0.0163
1993 Legako #2, TX	82.1	11.3	0.2155
1993 Northwood, ND	86.7	12.1	0.1405
1994 Northwood 2525, ND	98.5	3.7	0.7812
2014 2A, ND	96.1	7.2	0.5110
2014 18B, ND	97.1	8.9	0.2349
2014 27B, ND	87.3	11.9	0.0674
2014 29C, ND	92.7	5.5	0.1178
2014 35B, ND	99.5	21.9	0.0849
2014 44A, ND	99.5	37.1	0.5781
2014 55A, SD	86.9	22.0	0.2849
2014 64A, SD	99.0	14.3	0.6467
2014 71E, SD	94.4	13.5	0.7792
2014 73A, ND	99.0	24.0	0.8951
2014 77A, ND	97.9	21.5	0.0881

Table C1. Mean percent infection by *Plasmopara halstedii* isolate of inoculated, nontreated control seed and azoxystrobin treated seed at 10 ug ai/seed and significance of effect in mean percent infection between greenhouse rooms (<0.05 when comparing rooms) (continued).

Isolates	Mean % Infection		Effect Between Greenhouse Rooms
	0 ug ai/seed	10 ug ai/seed	
2014 84A, ND	95.3	17.2	0.8090
2014 91A, ND	99.4	14.0	0.0584
2014 93B, ND	94.7	26.0	0.0584
2014 94C, ND	98.4	14.1	0.7830
2014 97B, ND	99.3	11.2	0.4090
2014 102A, ND	99.3	15.1	0.6825
2014 105E, ND	90.5	19.0	0.5476
2014 Grandin, ND	96.6	8.4	0.1687
2014 Hughes 5-7, SD	98.9	16.2	0.8261
2014 Rothsay 3, MN	95.6	32.6	0.0156
2014 Staples, MN	99.5	20.3	0.9390
2015 1C, ND	90.3	20.2	0.6006
2015 8B, ND	96.9	9.5	0.5720
2015 12C, ND	93.0	17.8	0.2521
2015 18B, ND	89.8	18.8	0.9601
2015 25A, ND	89.0	8.4	0.0454
2015 26A, ND	90.5	10.4	0.9972
2015 27A, ND	93.1	14.0	0.8053
2015 50A, ND	98.4	10.8	0.0003
2015 54A, ND	88.2	16.9	0.0155
2015 Brookings, SD	91.3	8.6	0.5631
2015 Carrington, ND	96.6	11.3	0.3719
2015 Casselton C, ND	82.1	8.7	0.0774
2015 Colfax C, ND	91.4	3.3	0.0982
2015 Grand Forks B, ND	98.0	15.4	0.5438
2015 Hazel A, MN	94.9	13.5	0.0771
2015 Langdon A, ND	96.9	7.2	0.0848
2015 Potter County, SD	87.4	6.5	0.0993
2015 Pumpjack E, NE	98.5	10.3	0.3405