PARASITIC FITNESS OF SDHI-SENSITIVE AND -RESISTANT ISOLATES OF *ALTERNARIA SOLANI*

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Early blight of potato (*Solanum tuberosum* L.) is caused by *Alternaria solani* Sorauer. The single-site mode of action chemistries of the succinate dehydrogenase inhibitors (SDHIs) and quinone outside inhibitors (QoIs) have been widely used for early blight control but resistance has developed rapidly to a number of fungicide chemistries. QoI resistance in *A. solani* has been attributed to the F129L mutation, or the substitution of phenylalanine with leucine at position 129. Resistance to SDHI fungicides in *A. solani* is conferred by five known point mutations on three *AsSdh* genes. Over 1,300 *A. solani* isolates collected from 11 states in 2013 through 2015 were characterized for the presence of mutations associated with QoI and SDHI resistance through real-time, SDH multiplex, and mismatch amplification analysis (MAMA) polymerase chain reaction (PCR). Approximately 95% of isolates collected from 2013 to 2015 possessed the F129L mutation. Additionally, 95% of the *A. solani* population was determined to be SDHI-resistant, with the most prevalent mutation being on the *AsSdhC* gene. All *A. solani* isolates collected that were characterized as possessing the D123E mutation, or the substitution of aspartic acid for glutamic acid at position 123 in the *AsSdhD* gene, were evaluated for boscalid and fluopyram sensitivity in vitro. Furthermore, 15 isolates characterized as being SDHI-sensitive or -resistant, including five D123E-mutant isolates, were evaluated in vivo for percentage disease control to boscalid and fluopyram. Sensitivity of D123E-mutant isolates to fluopyram ranged from 0.8 to 3 µg/ml, and were found to be sensitive or only slightly higher than those of baseline isolates, ranging from 0.1 to 0.6 µg/ml. Disease control of all five D123E isolates evaluated in vivo was reduced significantly with the application of fluopyram compared to SDHI-sensitive isolates. Fitness was compared among 120 SDHI-sensitive and -resistant *A. solani* isolates using the parameters of spore germination and mycelial growth in vitro and aggressiveness in vivo. Spore

germination and mycelial growth between SDHI-sensitive and -resistant isolates was not significantly different. However, D123E-mutant isolates were significantly more aggressive in in vivo assays compared to other SDHI-resistant and SDHI-sensitive isolates. These results illustrate the importance of implementing fungicide resistance management strategies.

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INTRODUCTION AND LITERATURE REVIEW

Introduction

Early blight, caused by the fungal pathogen *Alternaria solani* Sorauer, is an important chronic foliar disease of potato (*Solanum tuberosum* L.) throughout most areas of production. Progressive and premature defoliation decreases photosynthesis and reduces the storage of tuber assimilates, such as starch. Tubers inoculated through wounds at harvest can also develop sunken lesions in storage and lead to quality reduction (Rotem, 1994). Yield losses have been shown to reach above 30%, significantly reducing the profitability of potato production (Franc and Christ, 2001). *A. solani* is considered to be a difficult pathogen to control because it is widespread and few cultivars possess resistance (Pasche and Gudmestad, 2008). Therefore, a primary method of controlling this pathogen is the application of foliar fungicides (Pscheidt and Stevenson, 1988).

The single-site mode of action chemistries of the succinate dehydrogenase inhibitors (SDHIs) and quinone outside inhibitors (QoIs) have been widely applied for early blight control, but resistance has developed rapidly. The biochemical mode of action is similar in these two classes, as both inhibit the mitochondrial respiration process, SDHIs at complex II and QoIs at complex III (Bartlett et al. 2002; Avenot and Michailides, 2010). The QoI fungicide azoxystrobin was fully registered in 1999, but by 2001, reduced disease control was detected in commercial potato fields (Pasche et al. 2004). While QoI resistance in other pathogens is associated with the G143A mutation, reduced sensitivity in *A. solani* has been attributed to the F129L mutation, or the substitution of phenylalanine with leucine at position 129 (Pasche et al. 2005). The SDHI fungicide boscalid was registered for use in potato in 2005, but resistance was reported in *A. solani* isolates recovered from Idaho in 2009 (Wharton et al. 2012) and determined to be prevalent throughout much of the United States by 2012 (Gudmestad et al. 2013). Resistance to

SDHI fungicides in *A. solani* is conferred by five known point mutations on three *AsSdh* genes, two on the *AsSdhB* gene, one on *AsSdhC* gene, and two on the *AsSdhD* gene (Mallik et al. 2014).

Recent research has been exploring parasitic fitness penalties associated with fungicide resistance development. Fitness has been defined as the survival and reproductive success of an allele, individual, or group (Pringle & Taylor, 2002). Parameters of pathogenic fitness that are evaluated include the ability to grow, reproduce, and compete on a host, as well as aggressiveness and survival through repeated cycles. This study aims to characterize fitness penalties that may result from the presence of mutations conferring QoI and SDHI-resistance in *A. solani.* The specific objectives of the study are as follows:

1) Determine the spatial and temporal frequency distribution of mutations conferring QoI and SDHI resistance in *A. solani* across the United States.
2) Determine the impact of fluopyram on the frequency of the D123E mutation in *A. solani*.
3) Determine the parasitic fitness of fungicide-resistant and -sensitive isolates of *A. solani*.

Pathogen Introduction

The genus *Alternaria* contains some of the most common and diverse forms of Ascomycota fungi, including aggressive and opourtunistic plant pathogens (Weir, 1998). Genetic characteristics of *Alternaria* were first analyzed with a concentration on the formation of conidia (Elliot, 1917). *Alternaria* was described to be a distinct genera and it was proposed that the genus *Alternaria* be divided in groups of species based on similar spore structure. The genus Alternaria is classified in the Kingdom *Fungi*, Phylum *Ascomycota*, Family *Pleosporaceae*, and Order *Pleosporales* (Agrios, 1997). The genus *Alternaria* is classified in the Kingdom *Fungi*, Phylum *Ascomycota*, Family *Pleosporaceae*, and Order *Pleosporales* (Agrios, 1997). Studies have characterized *Alternaria* species further using the morphology of their large conidia, which are

solid, ovoid, multicelled, pale brown to brown in color, with longitudinal septa (Ellis, 1971). The conidia of *Alternaria solani* are quite similar to other *Alternaria* species, but are characterized as separate conidia borne on conidiaphores and uniquely beaked (Ellis, 1971). *A. solani* occurs on solanaceous plants including potato, tomato, eggplant, pepper and nightshade. Although *A. solani* appears to have only an asexual cycle, it does have a high amount of variation in morphology, physiology, genetic makeup, and pathogenicity among isolates (Chaerani and Voorrips, 2006). There is also a high degree of diversity among isolates when taking into account spatial distribution. A high degree of genetic diversity exists in *A. solani* isolates originating from the U.S.A., South Africa, Cuba, Brazil, Turkey, Greece, Canada, Russia, and China based on molecular markers and vegetative compatibility groups (Chaerani and Voorrips, 2006). Although there is high genetic variability in *A. solani*, there remains to be no conclusive evidence of distinct physiological races.

Disease Cycle and Symptomatology

Primary infection of potato foliage by *A. solani* is caused by inoculum provided by other infected hosts or by inoculum that over-seasons on infected plant debris. Lesions form, and spores produced are dislodged under conducive environmental conditions. Alternating wet and dry periods are most favorable for sporulation and dispersal (Franc and Christ, 2001). The spores produced by primary inoculum are responsible for secondary spread of the fungus to healthy tissue, which leads to an exponential increase of foliar infection characteristic of this polycyclic pathogen. Foliar symptoms of early blight on potato first appear as small, circular, dark brown or black spots on the foliage near the soil 2 or 3 days after infection, with sporulation occurring 3 to 5 days later (Rotem, 1994). Minimum and maximum temperatures for infection of potato by *A. solani* are 10 and 35 °C, respectively. The optimum temperature ranges from 20 to 30 °C

(Stevenson et al. 2001). Under high humidity, free moisture, and the previously stated range of temperatures, conidia germinate to form one or more germ tubes, which can penetrate the host epidermal cells directly by appressoria or enter the stromata or wounds. Senescing tissue and plants under stress from insect pressure and low nitrogen are especially susceptible to infection (Franc and Christ, 2001). Following sporulation, wind and splashing rain are the main mechanisms of dissemination, but splashing dispersal by overhead irrigation is also important (Rotem, 1994). As the lesion expands, the development of concentric rings of raised necrotic tissue appear, forming the target spot or bull's eye appearance, which is characteristic of early blight infection. As further progression continues, the lesions take on an angular appearance, due to limitation by the leaf veins, and a chlorotic halo can develop around each lesion (Stevenson, 1993). Severe foliar infection by the early to mid-bulking period can result in small tuber size, reduced tuber dry matter content, and yield loss. In addition, spores produced on the foliar lesions can also contaminate the soil and infect tubers wounded during harvesting (Venette and Harrison, 1973). Irregularly shaped, dark, sunken lesions develop on wounded tubers inoculated at harvest, but only become manifest after months of storage (Stevenson, 1993; Franc and Christ, 2001). At seasons end, *A. solani* can overwinter in a variety of ways, including on infected crop debris, infected tubers, as chlamydospores in soil, and on alternative solanaceous hosts such as hairy nightshade (*Solanum physalifolium*). Spores can survive freezing temperatures on or just below the soil surface as well (Rotem, 1968).

Disease Management

Host resistance. Currently, there are no commercially available potato cultivars that possess immunity to early blight. However, widely grown potato cultivars do vary in the degree of susceptibility to infection. It has been shown that planting a moderately resistant cultivar

reduces the amount of fungicide applications needed for early blight control throughout the season compared to planting a susceptible cultivar (Christ, 1991). The main determinant of the level of susceptibility is the age of the plant. Generally, early-maturing cultivars are more susceptible to early blight compared to late-maturing cultivars (Rotem and Feldman, 1965). However, in all cultivars, following the initiation of tuber production, susceptibility gradually increases and mature plants are very susceptible to infection (Rotem and Feldman, 1965; Johanson and Thurston, 1990).

Cultural practices. There are a number of cultural management practices that can be easily employed to reduce the amount of infection by *A. solani*. Since the primary overwintering strategy of the pathogen is on crop debris, following a sufficient rotation and eliminating infested plant debris can juristically reduce the amount of initial inoculum of the early blight fungus (Stevenson, 1993). Site selection for potato production is a very important factor as well. Planting in fields with adequate drainage and applying fertilizer to increase fertility will reduce plant stress and help maintain the potato crop in good health, reducing the susceptibility, and minimize losses due to early blight below economic levels (Pscheidt and Stevenson, 1988). Overhead irrigation schedules should be managed in order to minimize the duration of leaf wetness and, thereby, limiting the amount of free moisture that favors the fungus. Additionally, controlling volunteers and weed pressure in and around potato fields can help reduce inoculum (Stevenson, 1993). The infection of tubers can be mitigated through careful handling during the harvesting process to minimize wounding, avoiding harvesting in wet conditions, and storing tubers between temperatures of 10 to 13°C with high relative humidity and proper aeration (Stevenson, 1993; Secor and Gudmestad, 1999).

Chemical control. Although many management strategies should be employed together as part of an integrated approach to minimize early blight, the primary and most effective method of control is the application of foliar fungicides (Gudmestad et al. 2013). The most widely applied fungicides used for early blight management are the protectants mancozeb and chlorothalonil, applied at intervals of 7-10 days from early in the growing season to vine desiccation (Stevenson, 1993). The addition of these protectants in fungicide rotations or in mixtures is very advantageous, as they possess a multi-site mode of action that reduces the risk of fungicide resistance development and are also labeled for controlling late blight (*Phytophthora infestans*) (Rosenzweig et al. 2008). However, they provide insufficient control under high disease pressure and conducive environmental conditions (Gudmestad et al. 2013; Yellareddygari et al. 2016). In 1999, the strobilurin fungicide azoxystrobin received registration by the Environmental Protection Agency (EPA) and initially provided excellent early blight control at high disease levels. Strobilurins belong to the group of chemicals classified as Quinone outside inhibitor (QoI) fungicides (FRAC group C3; Fungicide Resistance Action Committee, 2013), which inhibit mitochondrial respiration at complex III (Bartlett et al. 2002). These fungicides possess a specific single-site mode of action, indicating a high risk for fungicide resistance development. In 2001, reduced-sensitivity to azoxystrobin was first observed in *A. solani* isolates recovered from Nebraska and North Dakota (Pasche et al. 2004; 2005). This reduced-sensitivity in *A. solani* has been attributed to a substitution of phenylalanine with leucine at position 129 (F129L) in *cytb*, which conveys a 12- to 15-fold loss in sensitivity in vitro to azoxystrobin and pyraclostrobin, thus resulting in a significant loss of disease control (Pasche et al. 2004). By 2006, *A. solani* isolates possessing the F129L mutation conferring resistance to QoI fungicides were widespread throughout much of the United States (Pasche and Gudmestad,

2008). Fortunately, more recently developed chemistries, such as pyrimethanil, difenoconazole, and metconazole, provide a high level of activity without any reported widespread resistance issues to date.

SDHI Fungicides

The target of succinate dehydrogenase-inhibiting (SDHI) fungicides is the mitochondrial complex II at either succinate ubiquinone reductase or succinate dehydrogenase (Sdh) in the respiratory chain of various phytopathogenic fungi (Avenot and Michailides, 2010). SDHIs inhibit fungal respiration by blocking the ubiquinone binding sites in the mitochondrial complex II. The Fungicide Resistance Action Committee [FRAC] lists 7 chemical subgroups with 8 active ingredients from 4 of these subgroups that are currently registered for use or under development that comprise the SDHIs (FRAC group C2; Fungicide Resistance Action Committee, 2013). SDHIs have the FRAC activity code 7 and are considered to be at medium to high risk for the development of fungicide resistance because of their single-site mode of activity (FRAC, 2016). Due to their unique mode and site of action, no evidence of cross-resistance has been observed between SDHIs and other chemical classes such as strobilurins, benzimidazoles, and anilinopyrimidines. Older SDHI chemistries such as carboxin and oxycarboxin, referred to as first generation SDHIs, have been sprayed internationally since the late 1960s, but only provided effective control of basidiomycete pathogens such as rusts (Kuhn, 1984). Resistance to carboxin has been reported in several pathogens throughout the last 25 years (Avenot and Michailides, 2010).

In contrast to first generation SDHIs, second generation SDHIs such as boscalid, penthiopyrad, and fluopyram have activity against a broad spectrum of pathogens on various crops (Avenot and Michailides, 2010; Gudmestad et al. 2013). When boscalid was registered in

the United States for use on potato in 2005, it initially provided excellent early blight control and replaced the QoI fungicides in the foliar fungicide program (Gudmestad et al. 2013). However, by 2009, selection pressure from frequent application of SDHI fungicides soon led to the development of resistance to boscalid in various pathogens, including *A. solani* (Fairchild et al. 2013). It was determined that there are two phenotypes of boscalid resistance that exist in *A. solani*, one conveying a moderate level of resistance with a 15- to 60-fold loss in sensitivity, and another conveying a high or very high level of resistance with >100-fold loss in sensitivity (Gudmestad et al. 2013). There are presently five known point mutations on three *AsSdh* genes that confer SDHI-resistance in *A. solani* (Mallik et al. 2014). A single point mutation at codon 278 on the *AsSdhB* gene leads to an exchange from histidine to tyrosine (H278Y) or arginine (H278R). The H278R exchange is associated with moderate boscalid and moderate penthiopyrad resistance, while the H278Y exchange is associated high boscalid and high penthiopyrad resistance (Mallik et al. 2014). A point mutation on the *AsSdhC* gene at codon 134 leads to an exchange from histidine to arginine (H134R), which is associated with high boscalid and very high penthiopyrad resistance. The other two point mutations conferring SDHI-resistance in *A. solani* occur on the *AsSdhD* gene, leading to the substitution of histidine for arginine (H133R) or aspartic for glutamic acid (D123E) at amino acid positions 133 and 123, respectively. The H133R mutation confers very high boscalid and moderate penthiopyrad resistance. The D123E mutation has been found to occur in a small number of *A. solani* isolates to date (Mallik et al. 2014).

Some differences have been demonstrated in the spatial distribution of mutations, as mutations in the *AsSdhB* gene are found to be the most common, as well as distributed in all states with resistance and isolates containing the H134R mutation seem to be predominant in the

upper Midwest (Mallik et al. 2014). Resistance to boscalid in *A. solani* recently has been determined to be widespread as approximately 75% of isolates assayed across all locations exhibited some level of resistance (Gudmestad et al. 2013). Although boscalid resistance in *A. solani* is prevalent, the degree of sensitivity among individual isolates has been demonstrated to vary widely (Gudmestad et al. 2013). Furthermore, resistance depends on the specific SDHI fungicide as well. While resistance in *A. solani* to boscalid and penthiopyrad has been widely reported, no resistance has been reported to fluopyram, registered in 2012, which has been observed to control early blight in areas where high boscalid resistance is present (N. C. Gudmestad, *personal communication*). However, it is believed that fluopyram may bind at a different location in complex II than boscalid, and therefore, has a slightly different site of action and is unaffected by the mutations currently known to exist in *A. solani* (Gudmestad et al. 2013; Avenot and Michailides, 2010; Sierotzki and Scalliet, 2013).

Parasitic Fitness

The development and evolution of fungicide resistance in fungal populations is widely known to be dependent on the fitness of the resistance isolates, and has important implications for overall disease management (Chapara et al. 2011). Fitness can be defined as the survival and reproductive success of an allele, individual, or group. Parameters of pathogenic fitness include the ability to grow, reproduce, and compete on a host, as well as pathogenicity (Pringle and Taylor, 2002). Fitness also can be defined as the ability of an organism to contribute to the future gene pool and has been described as the progeny-to-parent ratio over a specific period of time. Parasitic fitness, however, is defined as the relative ability of a parasitic genotype or population to persist successfully over time (MacHardy et al. 2001). One of the most critical factors affecting the evolution of resistance to fungicides is the parasitic fitness of resistant isolates

(Dekker, 1981). Parasitic fitness is a quantifiable evaluation and can be measured in a variety of ways such as reproductive rate, rate of multiplication, infection efficiency, and disease severity (Karaoglanidis et al. 2011).

Fitness costs of plant pathogens are assessed in terms of both predicted and realized fitness (Antonovics and Alexander, 1989). Predicted fitness measurements include spore germination, mycelial growth, germ tube length, incubation period, aggressiveness, and spore production. Realized fitness, however, refers to competitive experiments, or assessing the impacts of fitness on the dynamics of competition between resistant and sensitive isolates under both laboratory and field conditions with mixed inocula (Karaoglanidis et al. 2001). Assessing the fitness of fungicide-resistant isolates relative to fungicide-sensitive isolates in any fungal pathogen is crucial in determining if resistant isolates will persist in the absence of selection pressure (Chapara et al. 2011). The progression of evolution of fungicide resistance would be slowed if fungicide-resistant isolates exhibited a cost, or fitness penalty, associated with lower parasitic or saprophytic fitness. In other words, resistant isolates displaying decreased spore germination in vitro or decreased aggressiveness in vivo relative to sensitive isolates would suffer a predicted fitness cost (Karaoglanidis et al. 2001). In contrast, the absence of a fitness penalty in the resistant pathogen population would indicate a stable resistance frequency in the absence of selection pressure or rapid resistance development and evolution in the presence of selection pressure (Karaoglanidis et al. 2011). However, it has been shown throughout multiple studies that the absence or presence of a fitness penalty is dependent upon the fungal species and the fungicide class (Karaoglanidis et al. 2001). A specific pathogen could also exhibit a predicted fitness penalty in vitro, but no fitness penalties in vivo. In contrast, there could be no fitness penalties observed in vitro, but a penalty that is only manifest in vivo. Since parasitic fitness of

an individual isolate of a pathogen is likely influenced by aggressiveness and the rate of spore production, and that these characteristics vary among isolates, fitness will also vary (Peever and Milgroom, 1995). Variations among parasitic fitness parameters may also be due to differences in the genetic background of the isolates tested.

Numerous studies have been conducted in recent years exploring parasitic fitness penalties associated with fungicide resistance development. However, research conducted to evaluate the relationship between fungicide resistance and parasitic fitness has resulted in many different and contradictory findings (Peever and Milgroom, 1995). Although the fitness of resistant strains in the absence of the fungicide is believed to play an essential role in shaping the outcomes of competition between fungicide-resistant and -sensitive strains, varying results in competition have been observed in different pathosystems. The differences in results among fitness studies and variation among similar studies conducted could be explained by the use of different fitness measures between studies and the sources of isolates used (Peever and Milgroom, 1995). Furthermore, there are variations in aggressiveness between individual isolates as well, and therefore, the assessment of fitness between isolates with difference in aggressiveness can lead to very different results.

The fitness of twenty metalaxyl-resistant and -sensitive isolates of *Phytophthora infestans* on potato were compared using measures of infection frequency (proportion of leaflets infected out of those inoculated), lesion area, and sporulation capacity (Kadish, 1988). Ten metalaxyl-resistant and ten metalaxyl-sensitive isolates were used in the assessment of all three measures and the proportion of resistant sporangia recovered from the population after mixed resistant and sensitive inoculations was measured. With competitive inoculations in this study, it was found that both resistant and sensitive isolates exhibited a large amount of variation and that

metalaxyl-resistant isolates of *P. infestans* had produced significantly higher lesion area, but did not differ from sensitive isolates in regard to sporulation capacity and infection efficiency (Kadish and Cohen, 1988). Although there were significant differences in fitness among metalaxyl-resistant and -sensitive isolates in noncompetitive experiments in vitro, no significant fitness penalties were demonstrated in competitive inoculations in vivo (Kadish and Cohen, 1988). This study shows the importance of conducting both in vitro and in vivo assays for fitness, and that competitive inoculations in vivo may not show any significant differences in fitness that were shown in a laboratory setting.

Competitive parasitic fitness of mefenoxam-senstive and -resistant isolates of *Phytophthora erythroseptica* also have been assessed using inoculum/infestation ratios of resistant and sensitive isolates that were equally aggressive (Chapara et al. 2011). Prior to conducting the fitness study, *P. erythroseptica* isolate aggressiveness was evaluated on non-fungicide treated tubers using challenge inoculations. Two isolates were selected for field infestation and challenge inoculation trials that were nearly numerically identical in aggressiveness index; one mefenoxam-resistant and one mefenoxam-sensitive. Competitive parasitic fitness was then assessed using soil infestations of mixed populations of mefenoxam-resistant and -sensitive *P. erythroseptica* isolates (Chapara et al. 2011). When mefenoxam was applied and provided selection pressure, significantly more resistant isolates were recovered regardless of resistant/sensitive ratio applied. In the absence of mefenoxam selection pressure, the ratio of recovery of -sensitive and -resistant isolates was not significantly different at all four infestation ratios. Thus, mefenoxam-resistant isolates emerged as more competitively fit in the presence of selection pressure, and equally fit in the absence of selection pressure, than mefenoxam-sensitive isolates of *P. erythroseptica* (Chapara et al. 2011). Prior to

this competitive study, *in vitro* growth and reproduction of phenylamide-resistant and -sensitive isolates of *P. erythroseptica* was conducted (Porter et al. 2007). Twenty metalaxyl-resistant and twenty metalaxyl-sensitive isolates were selected and assessed for mycelial growth rate and oospore production. Following this, five metalaxyl-resistant and five -sensitive isolates were selected from the first forty and assessed for zoospore germination and zoospore production (Porter et al. 2007). Metalaxyl-resistant *P. erythroseptica* isolates had greater mycelial growth and oospore production than metalaxyl-sensitive isolates, but that metalaxyl sensitive isolates had significantly greater zoospore production than metalaxyl-resistant isolates, indicating a potential asexual fitness penalty (Porter et al. 2007). This is another example of a fitness penalty detected in vitro, but no fitness penalties detected in the field.

Competitive parasitic ability was also evaluated on metalaxyl-resistant isolates of *Phytophthora nicotianae* (Timmer et al. 1998). Different pairings of metalxyl-resistant and –sensitive isolates were prepared in five different proportions and inoculated onto sour orange seedlings grown in the greenhouse. At the end of the experiments, the percentage root rot, the percentage of infected roots, and the propagule densities were taken and the relative recoveries of resistant and sensitive isolates were determined. Additionally, a field study was performed also as part of the experiment in which propagule densities of resistant and sensitive isolates were compared (Timmer et al. 1998). In the greenhouse, the competitive ability of metalaxyl-resistant isolates varied with the isolate pairing and that resistant isolates did not appear to be any less fit than sensitive isolates, with some resistant isolates competing favorably with the sensitive isolates in the absence of metalaxyl application. In the field, the propagule densities of resistant isolates did not decline throughout the 2.5 year study, both in the presence

and absence of metalaxyl usage, and thus, resistant isolates appeared to be as fit as sensitive isolates.

Fitness and competitive ability of fungicide-resistant isolates of *Botrytis cinerea* has been the subject of extensive research in recent years, as the fungal pathogen has gained resistance towards many important active ingredients used in its control (Veloukas et al. 2014; Bardas et al. 2008). *B. cinerea* causes gray mold on many widely grown fruit and vegetable crops worldwide, and the main management tool is the application of fungicides (Veloukas et al. 2014). The fitness of anilinopyrimidine-resistant isolates compared to sensitive isolates collected from vegetable crops in Greece was assessed in a series of experiments (Bardas et al. 2008). The fitness parameters used in this initial study were mycelial growth, spore production in vitro and in vivo, virulence, osmotic sensitivity, and germination ability of conidia, with competitive ability in vitro and in vivo also being assessed. While results were demonstrated to be largely isolate dependent among the parameters assessed, the anilinopyrimidine-resistant isolates had significantly lower mean mycelial growth, and were osmotically more sensitive, than anilinopyrimidine-sensitive isolates. Mean spore production, virulence, and conidial germination between resistant and sensitive isolates was not significantly different (Bardas et al. 2008). With competitive ability, in vitro studies determined that in two of the four sensitive and resistant isolate pairs, the sensitive isolates became dominant over the resistant isolates after five culture cycles, and the same was shown in vivo on cucumber seedlings. However, among all fitness parameters used, of the 10 cyprodinil-resistant and 10 -sensitive isolates, the resistant isolates appeared to have similar fitness characteristics to those of the sensitive isolates, and conclusions were isolate dependent (Bardas et al. 2008).

Fitness and stability of pyraclostrobin- and boscalid-resistant field isolates of *Botrytis cinerea* also has been studied (Kim and Xiao, 2011). This study assessed fitness with pathogenic parameters of pathogenicity and virulence, and saprophytic fitness parameters of mycelial growth, spore production, and osmotic sensitivity in vitro, and also competitive ability on apple fruit. It was demonstrated that there was great variability in mycelial growth, spore production, and sensitivity to osmotic stress among isolates sensitive to both pyraclostrobin and boscalid, as well as among isolates resistant to pyraclostrobin, boscalid, or both (Kim and Xiao, 2011). However, when compared as phenotype groups, there were no significant differences in average mycelial growth, spore production, or osmotic sensitivity. Regardless of fungicide, all inoculated fruit developed symptoms, indicating pathogenicity was not significantly different between sensitive and resistant isolates. There was also no difference in virulence among phenotype groups. In competitive inoculations, pyraclostrobin-resistant isolates were detectable in the populations regardless of ratio applied, but proportion observed was much lower than inoculated after being cycled for four generations. There were no boscalid-resistant isolates detected after four generations on the apple fruit, as boscalid-resistant isolates were outcompeted by pyraclostrobin-boscalid-resistant isolates (Kim and Xiao, 2011). Overall, there were no fitness penalties shown, except for boscalid-resistant isolates being overcome by isolates possessing resistance to both fungicides. None of the pyraclostrobin- or boscalid-resistant isolates lost resistance in the absence of fungicide pressure, thus, resistance was also demonstrated to be stable.

Other studies have also assessed the fitness and competitive ability of *B. cinerea* isolates with dual resistance to QoI and SDHI fungicides and presented conflicting outcomes (Veloukas et al. 2014). Parameters used were mycelial growth, conidial germination, and sclerotia

production in vitro, as well as aggressiveness and spore production in vivo. Isolates with SDHI resistance conferred by a mutation on the *SdhB* gene and QoI resistance by the G143A mutation on cytochrome b (*cyt*b) were assessed through these parameters, and in competitive experiments as well, in different combinations. Results determined that isolates with single resistance to QoIs did not suffer any fitness cost, but that isolates possessing dual resistance with different *SdhB* mutations and the G143A mutation suffered significant fitness penalties (Veloukas et al. 2014). Isolates possessing the histidine to arginine exchange at codon 272 in the *SdhB* gene (H272R mutation), conferring SDHI resistance, had aggressiveness and spore production similar to that of sensitive isolates, but lower mycelial growth and sclerotia production. Other *SdhB*-mutant isolates showed significantly lower values in all fitness parameters tested (Veloukas et al. 2014). Other similar studies have reported that isogenic strains of *B. cinerea* possessing several *SdhB* mutations showed a high fitness cost compared with the wild-type strain (Laleve et al. 2014).

Additional fitness studies have been conducted with *Alternaria* species that possess fungicide resistance. The fitness of *Alternaria alternata* isolates resistant to QoI fungicides was studied using parameters of mycelial growth and spore production in vitro, as well as aggressiveness and spore production in vivo (Karaoglanidis et al. 2011). Competitive experiments were also conducted using mixed-isolate inocula of four pairs of one resistant and one sensitive isolate at five ratios, and inoculated onto pistachio leaves continuing through four disease cycles. Observed ratios were compared to inoculation ratios of QoI-resistant and -sensitive isolates through real-time PCR. All predicted fitness assays of mycelial growth and spore production in vitro, as well as aggressiveness and spore production in vivo, QoI-resistant isolates were not significantly different from -sensitive isolates. In competitive experiments, resistant isolates dominated the population regardless of ratio inoculated in two of

the four isolate pairs, with sensitive isolates disappearing in the fourth disease cycle. However, in the other two isolate pairs, sensitive isolates were better competitors, but resistant isolates were still detected in the fourth disease cycle (Karaoglanidis et al. 2011). In conclusion, the four QoI-resistant isolates appeared to have fitness characteristics similar to those of the -sensitive isolates with regard to the parameters studied. Within competition experiments between the pairs of QoI-resistant and -sensitive isolates, the outcomes were isolate dependent, but this may be due to unequal aggressiveness between the resistant and sensitive isolates of *A. alternata* used. These results show a lack of any fitness penalty detected in QoI-resistant isolates of *A. alternata.*

Competitive fitness of *A. solani* isolates with reduced sensitivity to QoI fungicides was determined to be similar to QoI-resistant isolates of *A. alternata* when 45 QoI-sensitive isolates of *A. solani* were compared to 57 QoI-resistant isolates using both in vitro and in vivo methods (Pasche and Gudmestad, 2008). In vitro spore germination assays revealed that QoI-sensitive isolates had a higher mean percentage spore germination compared to -resistant isolates, indicating a potential fitness penalty. However, in vivo experiments demonstrated that the mean percentage of disease severity on non-treated greenhouse-grown tomato plants inoculated with QoI-sensitive isolates was significantly lower than the infection produced on tomato plants inoculated with QoI-resistant isolates (Pasche and Gudmestad, 2008). As shown, there may be a potential spore germination fitness penalty, but the increased severity of resistant isolates in vivo suggests that this penalty does not significantly impact realized fitness (Pasche and Gudmestad, 2008). Although no significant fitness penalties were characterized in QoI-resistant isolates of *A. solani* or *A. alternata*, these studies are still important. Fungicide resistance has emerged within the populations of each of these pathogens to not only QoI fungicides, but also SDHI fungicides, and employing anti-resistant management strategies to delay the selection of QoI-resistant strains

is critical. The level of success and the implementation of these management strategies depends on the knowledge of fitness of the resistant strains (Mikaberidze et al. 2014).

Although the existence of fitness penalties in fungicide resistant isolates of phytopathogenic fungi vary depending on the pathogen, fungicide class, and genetic background of the pathogen population, the conclusions from the assessment of fitness, both predicted and realized, have important implications on disease management (Skylakakis, 1981). There is limited information on the effect of any SDHI mutations on the fitness of SDHI-resistant fungal plant pathogens, and nothing to date is known about the fitness of SDHI-resistant *A. solani* isolates. However, since both QoI and SDHI resistance is prevalent in *A. solani*, the question of pathogenic fitness becomes important for a variety of reasons. It is essential to continue this research, as significant fitness penalties in *A. solani* isolates possessing dual resistance to both QoI and SDHI fungicides may exist. One popular and widely used practice to delay the development of fungicide resistance is the practice of tank-mixing, or mixing chemicals with different modes of action in fungicide applications (Mikaberidze et al. 2014). Fungicide mixtures released by the agrochemical industry often contain low-risk fungicides, to which fungal pathogens are fully sensitive, together with high-risk fungicides known to be prone to fungicide resistance. However, whether these mixtures provide adequate disease control while minimizing the risk for fungicide resistance development is dependent upon the existence of fitness penalties in fungicide-resistant isolates, and thus, is the basis of assessing the usefulness of a mixture (Mikaberidze et al. 2014). In the absence of fitness penalties, application of a mixture of high-risk and low-risk fungicides will select for resistance, and the resistant strain will then dominate the pathogen population, rendering the high-risk fungicide nonfunctional in the mixture. However, if sufficiently high fitness costs are found, the high-risk fungicides can be used

effectively for an extended period of time (Mikaberidze et al. 2014; Skylakakis, 1981). In assessing SDHI and QoI activity in mixtures and alternations to further delay resistance, determining fitness penalties associated with both QoI and SDHI resistance development in *A. solani* will be critical.

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CHAPTER 1. SPATIAL AND TEMPORAL FREQUENCY DISTRIBUTION OF MUTATIONS CONFERRING QOI AND SDHI RESISTANCE IN *ALTERNARIA SOLANI* ACROSS THE UNITED STATES

Abstract

The application of succinate dehydrogenase inhibiting (SDHI) and quinone outside inhibiting (QoI) fungicide chemistries is a primary tactic in the management of early blight of potato, caused by *Alternaria solani*. Resistance to QoIs in *A. solani* has been attributed to the F129L mutation, while resistance to SDHIs is conferred by five different known point mutations on three *AsSdh* genes. A total of 1,323 isolates were collected from 2013 through 2015 across 11 states to determine spatial and temporal frequency distribution of these mutations. A real-time polymerase chain reaction (PCR) was used to detect the presence of the F129L mutation. Molecular detection of SDHI-resistant isolates was performed using SDH multiplex PCR specific for point mutations in *AsSdhB*, *AsSdhC*, or *AsSdhD* genes and mismatch amplification analysis (MAMA)-PCR detecting the point mutations in *AsSdhB*. Previous work in our research group determined that substitutions of histidine for tyrosine (H278Y) or arginine (H278R) at codon 278 on the *AsSdhB* gene were the most prevalent mutations, detected in 46 and 21% of *A. solani* isolates collected in 2011, respectively, and uniformly distributed among six sampled states. In contrast, the substitution of histidine for arginine (H134R) at codon 134 in the *AsSdhC* gene, which was detected in only 7.5% of isolates recovered in 2011, was the most prevalent mutation in 2013 through 2015, identified in 36% of isolates. Substitutions of histidine for arginine (H133R) at codon 133 and aspartic acid for glutamic acid (D123E) at codon 123 in the *AsSdhD* gene were detected in 15 and 1.5% of isolates collected in 2011, respectively, and appeared to be region-specific. In 2013 through 2015, the H133R mutation was similarly

detected in 16% of isolates, but *A. solani* isolates possessing the D123E mutation comprised 12% of the population by 2015 and were recovered across a wide range of states. Overall, SDHI- and QoI-resistant isolates were detected at high frequencies across all years, with evidence of significant spatial variability. Future research will investigate if these results are due to differences in parasitic fitness.

Introduction

Early blight of potato (*Solanum tuberosum* L.), caused by *Alternaria solani* Sorauer, is a chronic foliar disease of potato present every growing season throughout many potato production areas and can cause significant yield reductions (Franc and Christ, 2001). Since most currently grown potato cultivars are susceptible to early blight, the primary method of disease control is the application of foliar fungicides. Although mancozeb and chlorothalonil remain the most frequently applied protectant fungicides for the control of early blight, they provide insufficient control under high disease pressure (Gudmestad et al. 2013). The single-site mode of action chemistries of the succinate dehydrogenase inhibitors (SDHIs) and quinone outside inhibitors (QoIs) have been widely used for early blight control but resistance has developed rapidly to a number of fungicide chemistries. The biochemical mode of action is similar in these two classes, as they both inhibit the mitochondrial respiration process, SDHIs at complex II and QoIs at complex III (Stammler et al. 2007).

The QoI fungicide azoxystrobin was introduced in 1999 and initially provided excellent early blight control, but reduced disease control was observed in *A. solani* by 2001 (Pasche et al. 2004). While QoI resistance in other pathogens is associated with the G143A mutation, or the substitution of glycine with alanine at position 143 in the cytochrome b (*cytb*) gene, reduced sensitivity in *A. solani* is attributed to the F129L mutation, or the substitution of phenylalanine

with leucine at position 129 (Pasche et al. 2005). The F129L mutation conveys a moderate level of resistance to QoI fungicides such as azoxystrobin and pyraclostrobin, resulting in a 12- to 15-fold reduction in sensitivity. In a survey conducted from 2002 through 2006, the prevalence of F129L-mutant *A. solani* isolates was 96.5%, predominating the population of isolates collected across 11 states (Pasche and Gudmestad, 2008).

In 2005, the SDHI fungicide boscalid was registered for use on potato and provided effective early blight control after the development of resistance in *A. solani* to azoxystrobin. Unfortunately, *A. solani* isolates found to be insensitive to boscalid were recovered in Idaho in 2009 (Wharton et al. 2012). Resistance to SDHI fungicides in *A. solani* is conferred by five known point mutations on three *AsSdh* genes; two on the As*SdhB* gene, one on As*SdhC* gene, and two on the As*SdhD* gene (Mallik et al. 2014). Similar to mutations identified in *Alternaria alternata*, each mutation identified in the three target-encoding genes in *A. solani* can independently confer resistance to SDHI fungicides (Avenot et al. 2008; Mallik et al. 2014). Point mutations on the *AsSdhB* gene leading to an exchange from histidine to tyrosine (H278Y) or arginine (H278R), respectively, at codon 278 are associated with moderate to high levels of resistance to boscalid and penthiopyrad. A point mutation in the *AsSdhC* gene leading to an exchange from histidine to arginine (H134R) at codon 134 confers very high boscalid and penthiopyrad resistance. Two additional point mutations on the *AsSdhD* gene leading to exchanges from histidine to arginine (H133R) at codon 133, and aspartic acid to glutamic acid (D123E) at codon 123, are also associated with high levels of resistance to boscalid and penthiopyrad (Mallik et al. 2014).

In addition to *A. solani*, mutations conferring SDHI resistance have been identified in many pathogens such as *Alternaria alternata*, *Botrytis cinerea*, *Corynespora cassiicola*,

Didymella bryoniae, and *Podosphaera xanthii*, (Avenot et al. 2008, 2010, 2012; Bardas et al. 2010; Ishii et al. 2011) among others. Several studies have suggested evidence of spatial variability of mutations in SDH subunits conveying resistance. For instance, mutations in the *BcSdhB* gene conferring SDHI resistance in *B. cinerea* were shown to be widely variable among production areas with five SDH mutations detected in isolates recovered from Greek strawberry fields and seven different mutations detected in isolates recovered from grapes in France (Konstantinou et al. 2015; Leroux et al. 2010). In the Greek strawberry population of *B. cinerea,* the H272R mutation was the most prevalent (Konstantinou et al. 2015), but in the French grape population, the H272Y was detected at the highest frequency (Leroux et al. 2010). *Botrytis cinerea* isolates possessing the H272R and H272Y mutations were also predominate in blackberry and strawberry fields in South and North Carolina, with the two mutations appearing at a ratio of 2:1, respectively (Li et al. 2014).

In SDHI-resistant isolates of *A. alternata* recovered from pistachio in California, mutations were detected in the *AaSdhB, AaSdhC,* and *AaSdhD* genes, but isolates possessing mutations in the *AsSdhD* gene were detected at lower frequencies (Avenot et al. 2009; Avenot and Michailides, 2010). Mutations on all three SDH genes were also identified in isolates of *A. alternata* collected from peach orchards in South Carolina, with the H134R mutation on the *AaSdhC* gene found to be the most prevalent (Yang et al. 2015). In a survey of *A. solani* in 2011, isolates possessing mutations in the *AsSdhB* gene were recovered at the highest frequencies and uniformly distributed among six samples states, while isolates possessing mutations on the *AsSdhC* and *AsSdhD* genes were recovered at lower frequencies and appeared to be region specific (Mallik et al. 2014). For example, *A. solani* isolates possessing the H278Y mutation were collected in all six sampled states and constituted 46% of the population, while isolates

with the H134R mutation constituted 7.5% of the population and were only recovered in North Dakota and Idaho. Furthermore, the D123E mutation was only detected in a single isolate which was recovered from Nebraska. Despite variation in spatial distribution of mutations associated with fungicide resistance development in the aforementioned pathogens, very little is known about temporal variation throughout multiple years.

A key element of fungicide resistance management is the continued monitoring of resistance development in fungal populations over time, which can allow predictions of pathogen behavior, detect shifts in pathogen sensitivity, assess efficacy of fungicide regimes, and recommend effective resistance management tactics (Thomas et al. 2012). In addition to collecting a spatially and temporally diverse *A. solani* population from potato, the objectives of this study were to (i) determine the overall prevalence of SDHI and QoI resistance in *A. solani*; (ii) determine temporal frequency of mutations conferring SDHI and QoI resistance from 2011 through 2015; and (iii) determine spatial frequency of mutations conferring SDHI and QoI resistance across potato production areas in the United States.

Materials and Methods

***A. solani* isolate collection**. A total of 1,323 *A. solani* isolates collected from 2013 through 2015 were obtained from foliar and tuber samples submitted to our laboratory from potato producing areas in 11 states across the United States including North Dakota, Minnesota, Nebraska, Texas, Colorado, New Mexico, Idaho, Washington, Michigan, Illinois, and Wisconsin (Table 1.1). Approximately 15 to 20 infected leaves were randomly collected from each potato field, stored with a damp paper towel in a plastic bag, and mailed directly to our laboratory. Three to five infected leaflets from each field were stored in a herbarium press for preservation of the original collection. *A. solani* isolates were obtained by transferring a small portion of plant

tissue from the margin of early blight lesions directly to solid 1.5% water agar media and grown at room temperature (22 ± 2°C) for 4 days until conidia were produced (Holm et al. 2003). Following this, a single conidium was transferred to a solid clarified V8 media (CV8) (Campbell's V8 juice, 100 ml; $CaCO_3$, 1.5g; agar, 15 g; and distilled water, 900ml) amended with 50 mg of ampicillin per milliliter. Cultures were incubated under continuous fluorescent light for 7 to 10 days and examined for the presence of *A. solani* conidia (Pasche et al. 2004). Long-term storage of individual isolates was achieved by filling two small, screw-top centrifuge tubes per isolate with 4-mm diameter plugs of media with fungal mycelia and conidia excised using a sterilized cork borer (Fonseka and Gudmestad, 2016). The loosely capped tubes containing plugs were placed within silica gel in a closed container for 2 to 3 days to remove moisture from the media and caps were then tightened, sealed with Parafilm, and tubes placed in an ultrafreezer at a temperature of -80°C.

Table 1.1. Origin and collection year for *Alternaria solani* isolates characterized for presence or absence of mutations associated with succinate dehydrogenase inhibitor (SDHI) and quinone outside inhibitor (QoI) resistance.

	2011[z]	2013	2014	2015	Total
Colorado	2	80	0	26	108
Idaho	19	1	17	3	40
Illinois	0	19	0	0	19
Michigan	0	16	16	0	32
Minnesota	12	51	38	181	282
Nebraska	18	47	58	138	261
New Mexico	0	37	0	0	37
North Dakota	10	83	80	70	243
Texas	6	86	86	129	307
Washington	0	6	0	0	6
Wisconsin	0	40	0	15	55
Total	67	466	295	562	1390

[z] 2011 results via Mallik et al. 2014.

DNA extraction. DNA was extracted from all isolates using a modified cetyltrimethylammonium bromide (CTAB) method (Mallik et al. 2014; Stewart and Via, 1999).

First, mycelia and spores were scraped from a 7-day old culture of *A. solani* into an autoclaved mortar and ground to fine powder with liquid nitrogen. Approximately 100mg of powder was transferred immediately into a tube of lysing matrix A (MP Biomedicals LLC, OH) consisting of 750 µl of Carlson lysis buffer (100 mM Tris HC1 [pH 9.5], 2% CTAB, 1.4 M NaCl, 1% PEG 8000, and 20 mM EDTA) supplemented with 2% β-mercaptoethanol. The tube was placed in a FastPrep instrument (MP Biomedicals) and subjected to agitation at a speed of 6.00 m/s for 40 s to facilitate the homogenization of the mycelia and spore mixture. The sample was incubated in the tube at 75°C for 40 min with inversions at intervals of 10 to 15 min followed by a centrifugation at a speed of 14,000 × g for 10 min (Mallik et al. 2014). The supernatant was removed and placed immediately into a new tube. Nucleic acids were extracted in the aqueous phase by adding an equal volume of phenol/chloroform/isoamyl alcohol, 25:24:1(vol/vol) (Sigma-Aldrich, St. Louis, MO). Genomic DNA was then precipitated with an equal volume of isopropanol and washed with 95% ethanol. Finally, the genomic DNA was reconstituted in a glass-distilled RNA-ase-DNAase-free water (Teknova Inc., Hollister, CA) at a final concentration of 10 ng μl^{-1} and RNA-ase (0.2 µg) (Qiagen Inc. Valencia, CA) was added.

Detection of the F129L mutation. A real-time PCR-hybridization assay was used to detect the single-base-pair F129L mutation associated with reduced sensitivity to QoI fungicides in *A. solani* (Pasche et al. 2005). The PCR reaction was carried out using the LightCycler thermocycler (Roche) in glass capillaries in a final volume of 20 ml containing 1× FastStart DNA Master Hybridization Probes (Roche), 2mM MgCl2 (Roche), 0.5 µM AS-5F forward primer, 2.5 µM AS-5R reverse primer, 0.2 µM sensor (Asol-FL) and anchor (Asol-R640) probes, and 1 ng of DNA. After an initial denaturation step of 95°C for 10 min, PCR was run for 45 cycles using the following conditions: denaturation (95°C, 10 s, ramp rate 20°C/s), annealing (58

to 50°C, 10 s, ramp rate 20°C/s, step size 1°C, acquisition mode: single), and extension (72°C, 8 s, ramp rate 5°C/s). After amplification, melting curves were generated at 95°C (10 s, ramp rate 20°C/s), 45°C (30 s, ramp rate 20°C/s), and 85°C (0 s, ramp rate 0.1°C/s, acquisition mode: continuous). After a final cooling step for 30 s at 40°C, melting curve analysis was performed. The real-time method includes amplification of a fragment of the cytochrome *b* gene coupled with simultaneous detection of the product by probe hybridization and analysis of the melting point of the DNA fragments. The sensor probe in the assay is designed to span the mutation site so that the single nucleotide mismatch position is at least 3 bp away from the sensor probe end. When the sensor probe is melted away from the amplification product, the matching probe-target DNA will separate at a higher melting point temperature than probes that are bound to DNA which contain destabilizing nucleotide mismatches. A specific melting point temperature can be obtained for each genotype in this manner (Pasche et al. 2005). As assessed, if the highest melting point temperature for the genotype is below 55°C, the DNA fragment of the isolates possess the F129L mutation, and thus, the isolate has reduced sensitivity to QoIs. If the highest melting point is above 55°C, it is said to be wild-type.

Molecular detection of SDHI-resistant isolates. SDHI mutations were detected using previously described Polymerase chain reaction (PCR) methods (Mallik et al. 2014). Briefly, MAMA primers previously developed to distinguish two mutations in the *AsSdhB* gene. Amplification with MAMAB1-F and MAMABM-R primers, developed for isolates with the H278R mutation, yielded a 127-bp amplification product on agarose gel. Amplification with the MAMAB1-F and MAMABR-R primers, developed for isolates possessing the H278Y mutation, yields a 127-bp amplification product. However, isolates possessing a mutation associated with SDHI-resistance in either the *AsSdhC* or *AsSdhD* gene show no amplification bands in either the

H278R or H278Y MAMA-PCR. For detection of these mutations, additional PCR assays are needed. SNPs in *AsSdhC* and *AsSdhD* genes confer the H134R and H133R, respectively, and are amplified as part of a multiplex PCR assay previously developed. A 235-bp amplification product along with either a 457-bp product or a 72-bp product were amplified if a mutation existed in *AsSdhC* or *AsSdhD* genes, respectively. A single amplification product with a 235-bp product alone confirmed that an isolate did not possess any of the mutations in the *AsSdhB*, *AsSdhC* or *AsSdhD* genes. The absence of any amplification product in the multiplex PCR assay indicated that there is a possible mutation in the *AsSdhB* gene, and this was tested with the previously described MAMA-PCR assay. MAMA-PCR assays were performed using 25-µl volume consisting of 20 ng of DNA, 1.5 mM MgCl2, 0.2 mM dNTP, 5µM each primer, and 1 U of Go Taq polymerase (Promega Corp.). Multiplex PCR also consisted of 25 µl, with 20 ng of DNA, 2 mM MgCl2, 0.2 mM dNTP, 5 µM SdhBSen-F, 5 µM SdhBSen-R, 3 µM SdhC-F, 3 µM SdhC-R1, 5 µM SdhD-F, and 5 µM SdhD-R1 primers, respectively, followed by 1 U of Go Taq polymerase (Promega Corp.). The MAMA-PCR was done in a Peltier thermal cycler, DNA engine (Bio-Rad, Hurcules, CA), with an initial preheat step of 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. A final extension at 72°C for 7 min was also added at the end of the program. The multiplex PCR program for amplifying *AsSdhC* or *AsSdhD* mutations was the same as above, except an annealing temperature of 58°C was used. An additional PCR assay was then conducted on isolates that did not amplify in either of the previously discussed multiplex or MAMA-PCR tests. This PCR reaction was done in a thermal cycler DNA engine, with an initial preheat step of 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min and performed using 23-µl

volume consisting of 20 ng of DNA, 1.5 mM $MgCl^2$, 0.5 mM dNTP, 5µM each primer, and 1 U of Go Taq polymerase (Promega Corp.). Isolates that yielded a 127-bp amplification product with this reaction possess the D123E mutation. All amplified products were separated by gel electrophoresis in 1.2% agarose gel (Mallik et al. 2014).

Genotype characterization. Each *A. solani* isolate collected was also characterized for genotype I or genotype II, differing by the presence or absence of an intron in the *cytb* gene (Leiminger et al, 2014). Standard PCR was performed for the characterization of each genotype in a thermal cycler DNA engine, with an initial preheat step of 98°C for 2 min followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, and extension at 72°C for 10 s, with a final extension at 72°C for 5 min and performed using 23-µl volume consisting of 20 ng of DNA, 1 mM $MgCl_2$, 0.5 mM dNTP, 5µM each primer, and 1 U of Go Taq polymerase (Promega Corp.). The reaction was the same for the detection of both genotypes except for the primers used. For the detection of genotype I, the forward primer As-Gf and the reverse primer As-Gr, were used, yielding a 214-bp amplification product if the isolate possessed genotype I (Leiminger et al. 2014). For the detection of genotype II, the forward primer As-5f and reverse primer As-5r were used (Pasche et al. 2005). All amplified products were separated by gel electrophoresis in 1.2% agarose gel. For genotype II, a 207 bp fragment was amplified, and for genotype I, a 214 bp fragment was amplified (Leiminger et al. 2014).

Statistical analysis. Log-linear models were used to analyze categorical data (Jiménez-Díaz et al. 2011). Two categories were used: (i) year: 2011, 2013, 2014, or 2015; and (ii) state: North Dakota, Minnesota, Nebraska, Texas, Colorado, Wisconsin, New Mexico, Washington, Michigan, Illinois, or Idaho. The CATMOD procedure in SAS software (Statistical Analysis System, Version 9.3; SAS Institute, Cary, NC) with log link was used. PROC CATMOD also

provided maximum likelihood analysis of variance (ANOVA) and estimates of maximum likelihood for the main effects of year and state.

Results

Determination of overall prevalence of mutations conferring SDHI and QoI resistance.

Across three years of *A. solani* isolate collection from 2013-2015, 1,323 isolates were collected across 11 states across the United States and combined with 67 isolates obtained previously across six states (Mallik et al. 2014). The total number of isolates characterized for the presence of mutations associated with fungicide resistance was 1,390 (Table 1.1). The percentage of *A. solani* isolates possessing mutations conferring SDHI resistance ranged from 90% to 99% across all years surveyed (Fig. 1.1). Overall frequency of SDHI-resistant isolates increased from 90% in 2011 to above 96% in each of the last three years of the survey from 2013 through 2015. The percentage of SDHI-sensitive isolates, or *A. solani* isolates found to possess none of the five characterized mutations on the three *AsSdh* genes, was below 10% in each year and ranged from 8% in 2011 to below 1% in 2015. Similarly, *A. solani* isolates possessing the F129L mutation conferring resistance to QoI fungicides were also dominant throughout all four years of collection (Fig. 1.2). In 2011, the percentage of isolates possessing the F129L mutation was 92%, while 99% of the isolates collected in 2015 possessed this QoI mutation. Overall, the frequency of *A. solani* isolates possessing mutations conferring QoI and SDHI resistance was greater in each of the last three years of collection compared to results from 2011 (Mallik et al. 2014). Furthermore, nearly 100% of *A. solani* isolates possessing mutations conferring SDHI resistance collected each year also possess the F129L mutation conferring QoI resistance, indicating that isolates resistant to both chemical classes are predominant in *A. solani* populations across the United States.

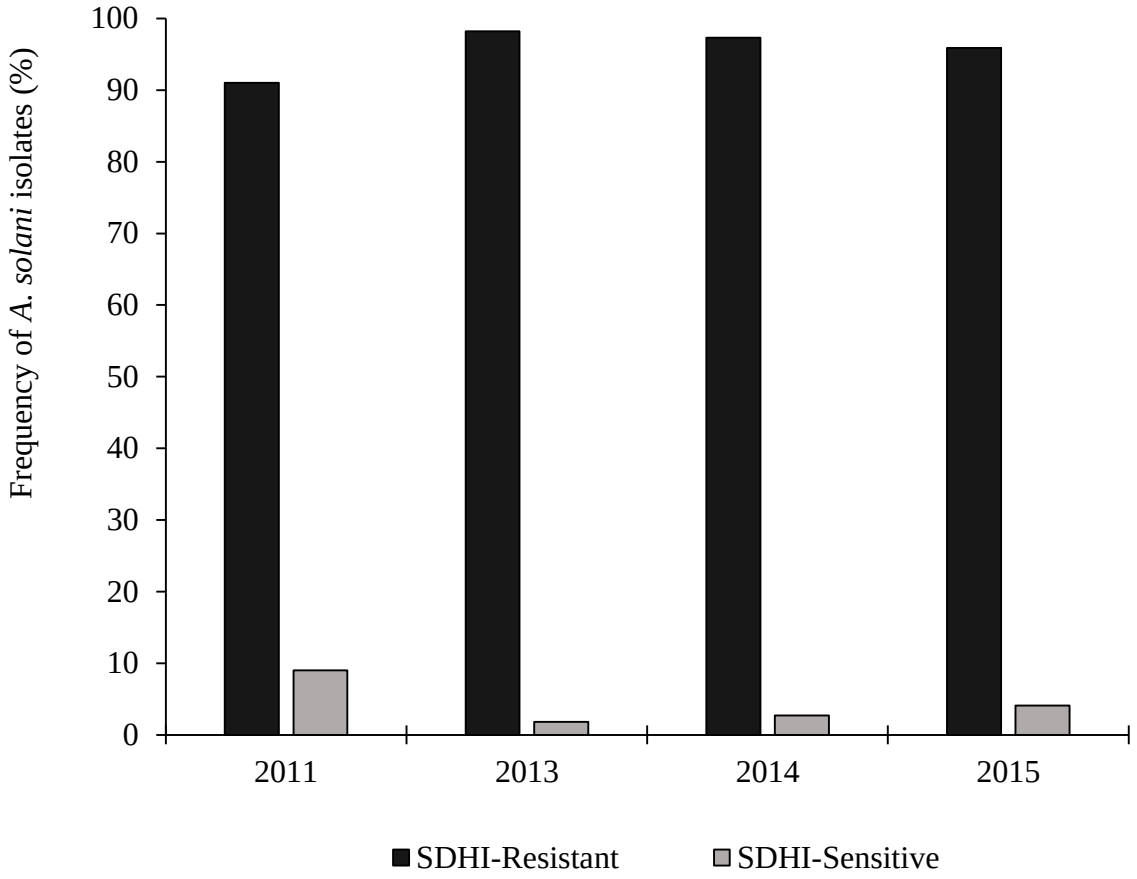

Fig. 1.1. Temporal frequency of *Alternaria solani* isolates possessing mutations conferring resistance to succinate dehydrogenase inhibiting (SDHI) fungicides. SDHI-sensitive isolates were characterized as possessing none of the five known mutations.

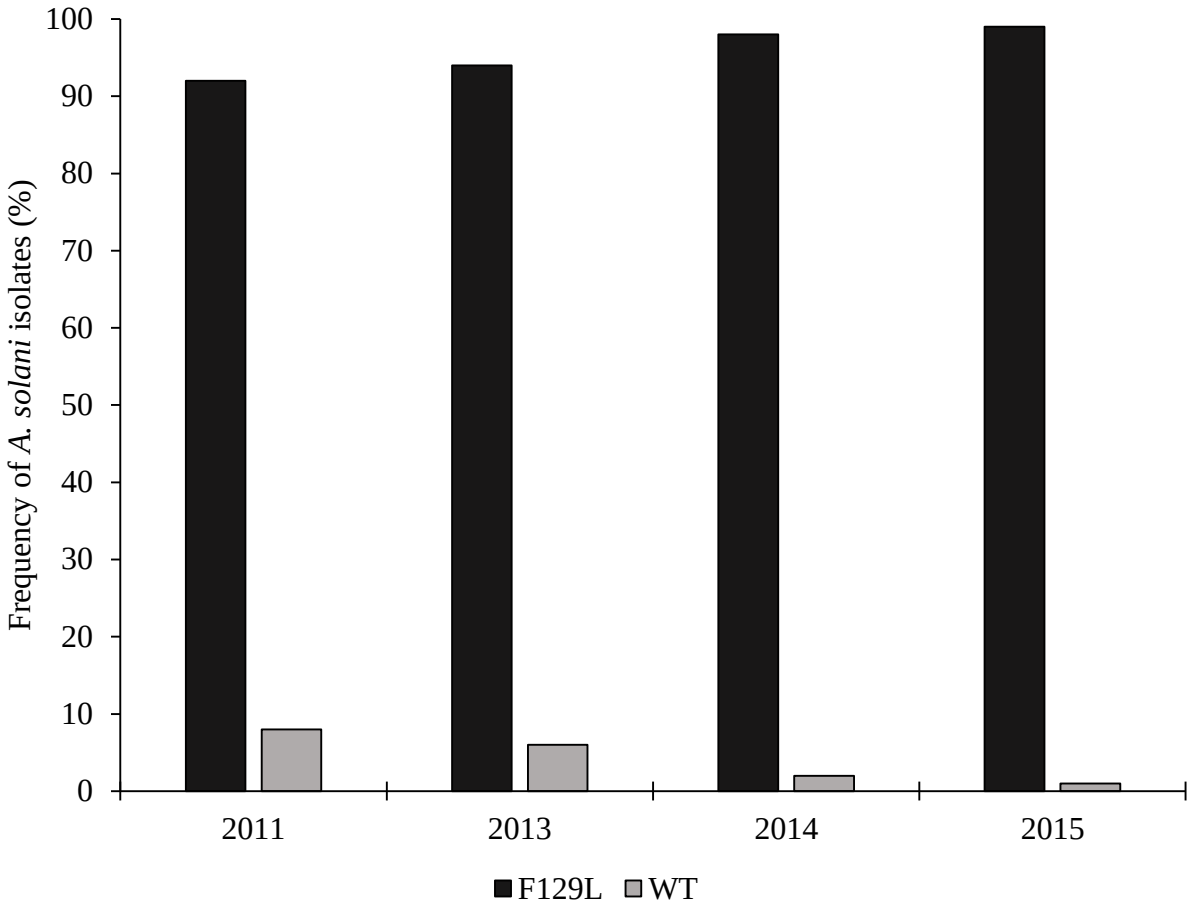

Fig. 1.2. Temporal frequency distribution of *Alternaria solani* isolates possessing the F129L mutation conferring quinone outside inhibitor (QoI) resistance. Wild-Type isolates are denoted "WT" and do not possess the F129L mutation.

Determination of temporal frequency distribution of mutations conferring QoI and SDHI resistance in *A. solani*. Maximum likelihood ANOVA from log-linear analysis determined that collection year had a significant effect (P=0.05) on the frequencies of mutations conferring SDHI and QoI resistance (Table 1.2). Collection year was shown to have significant effect (χ^2 = 96.46, P = <.0001) on the frequency of F129L-mutant *A. solani* isolates collected and there was a significant year × state interaction (χ^2 = 334.27, P = <.0001)(Table 1.2). Collection year was also shown to have significant effect on the frequency of *A. solani* isolates possessing the H278Y or H278R mutation on the *AsSdhB* gene conferring SDHI resistance (χ^2 = 193.09, and

20.20; P = <.0001, and P = 0.0002, respectively). In addition, there was also a significant year × state interaction having significant effect on frequency of both mutations. Similarly, collection year was shown to have significant effect on the frequency of *A. solani* isolates possessing the H134R mutation on the *AsSdhC* gene or the H133R mutation on the *AsSdhD* gene (χ^2 = 51.71, and 36.34; P = <.0001, and <.0001, respectively), with a significant year × state interaction, suggesting the combination of year and state have a significant effect on the frequency of these mutations. However, while collection year was shown to have a significant effect on the frequency of D123E-mutant isolates collected (χ^2 = 24.67, P = <.0001), there was no significant year × state interaction (χ^2 = 6.68, P = 0.2459), which indicates that year and state independently effect the frequency of D123E-mutants recovered (Table 1.2).

Table 1.2. Maximum likelihood analysis of variance from log-linear analysis for the effects of year and state on the frequency of mutations conferring resistance to succinate dehydrogenase inhibiting (SDHI) and quinone outside inhibiting (QoI) fungicides.

	Year			State			Year × State		
	df	χ^2	P > χ^{2a}	df	χ^2	P > χ^{2a}	df	χ^2	P > χ^{2a}
H278Y[b]	3	193.09	<.0001	9	87.80	<.0001	12	46.05	<.0001
H278R[b]	3	20.20	0.0002	8	29.88	0.0002	7	14.41	0.0444
H134R[b]	3	51.71	<.0001	8	103.09	<.0001	8	33.65	<.0001
H133R[b]	3	36.34	<.0001	9	52.04	<.0001	8	61.31	<.0001
D123E[b]	3	24.67	<.0001	5	13.50	0.0191	5	6.68	0.2459
F129L[c]	3	96.46	<.0001	10	597.57	<.0001	17	334.27	<.0001

[a] Significant if P < 0.05.
[b] Mutation confers resistance to succinate dehydrogenase inhibiting (SDHI) fungicides.
[c] Mutation confers resistance to quinone outside inhibiting (QoI) fungicides.

Analysis of maximum likelihood estimates from log-linear analysis for the main effects of year and state on the frequency of mutations conferring QoI and SDHI resistance in *A. solani* determined that the probabilities of collecting mutant isolates were significantly different among years (Table 1.3). The probability of collecting H278Y-mutant *A. solani* isolates was

significantly greater (P = <.0001) in 2015 compared to 2013 or 2014, whereas the probability of collecting H278R-mutants was significantly lower (P = <.0001) in 2015 and 2014 compared to 2013. The probabilities of collecting *A. solani* isolates possessing the H134R mutation on the *AsSdhC* gene were the highest in 2013 and 2015 (P = <.0001, and P=0.0005, respectively). H133R- and D123E-mutant *A. solani* isolates had significantly higher probability of detection in 2015 compared to other years (P = <.0001, and <.0001, respectively) (Table 1.3). Similarly, the probability of recovering F129L-mutant *A. solani* isolates was significantly higher in 2015 compared to other years of the survey (P = <.0001) (Table 1.3).

Table 1.3. Analysis of maximum likelihood estimates from log-linear analysis for the main effects of year on frequency of mutations conferring resistance to succinate dehydrogenase inhibiting (SDHI) and quinone outside inhibiting (QoI) fungicides among years.

Mutation	Year	Estimate	$P > \chi^{2a}$
H278Y[b]	2013	-0.5942	<.0001
	2014	0.4038	<.0001
	2015	1.1328	<.0001
H278R[b]	2013	0.8577	<.0001
	2014	-0.3273	0.3408
	2015	-0.8326	0.0303
H134R[b]	2013	0.9093	<.0001
	2014	0.2611	0.0856
	2015	0.5038	0.0005
H133R[b]	2013	-0.1234	0.3773
	2014	0.0164	0.9063
	2015	0.7549	<.0001
D123E[b]	2013	0.0098	0.9758
	2014	0.3283	0.2892
	2015	1.1850	<.0001
F129L[c]	2013	-0.1732	<.0001
	2014	-0.3180	<.0001
	2015	0.2601	<.0001

[a] Significant if P < 0.05.
[b] Mutation confers resistance to succinate dehydrogenase inhibiting (SDHI) fungicides.
[c] Mutation confers resistance to quinone outside inhibiting (QoI) fungicides.

Table 1.4. Analysis of maximum likelihood estimates from log-linear analysis for differences in the frequency of mutations conferring resistance to succinate dehydrogenase inhibiting (SDHI) and quinone outside inhibiting (QoI) fungicides among states.

Mutation	State	Estimate	$P > \chi^{2a}$	Mutation	State	Estimate	$P > \chi^{2a}$
	CO	-0.2701	0.4647		CO	-0.0874	0.7505
	IL	0.9571	0.0009		ID	0.0943	0.6798
	MN	0.3933	0.0227		MI	0.7783	0.0011
	ND	0.4421	0.0098		MN	1.1258	<.0001
H278Y[b]	NE	0.6934	<.0001	H133R[b]	ND	-0.9411	0.1485
	NM	1.4961	<.0001		NE	0.7875	<.0001
	TX	0.7904	<.0001		NM	-0.2480	0.6005
	WA	-1.6819	0.0642		TX	0.3728	0.1682
	WI	-1.0077	0.0004		WA	-0.9411	0.1485
	CO	0.3670	0.1755		CO	-0.5749	0.3597
	MN	-1.0215	0.0275		MI	-0.5236	0.2038
	ND	0.7290	0.0092	D123E[b]	ND	-0.2852	0.2838
H278R[b]	NE	-0.1052	0.7370		NE	0.2648	0.2455
	NM	0.3026	0.3951		TX	0.6177	0.0034
	TX	-0.6160	0.1117		CO	-0.0448	0.6672
	WA	-0.6782	0.2068		ID	-1.4365	<.0001
	WI	1.1449	<.0001		IL	-0.3675	0.0898
	CO	0.8977	<.0001		MI	-0.568	0.0014
	IL	-1.1867	0.0063	F129L[c]	MN	1.2092	<.0001
	MN	0.8389	<.0001		ND	0.7982	<.0001
H134R[b]	ND	1.1737	<.0001		NE	0.9726	<.0001
	NE	0.4454	0.0380		NM	0.299	0.0635
	NM	-2.7962	0.0019		TX	1.1615	<.0001
	TX	1.2564	<.0001		WA	-1.5201	<.0001
	WI	-0.4166	0.1294				

[a] Significant if $P < 0.05$.
[b] Mutation confers resistance to succinate dehydrogenase inhibiting (SDHI) fungicides.
[c] Mutation confers resistance to quinone outside inhibiting (QoI) fungicides.

Overall, from 2013-2015, *A. solani* isolates possessing mutations on the *AsSdhB* gene were collected at lower frequencies compared to 2011 (Fig. 1.3A; B). The frequency of *A. solani* isolates possessing the H278R mutation significantly decreased over time (Fig. 1.3B). The frequency of H278Y-mutant *A. solani* isolates was dynamic from year to year, increasing from 2013 to 2015. Despite this increase, *A. solani* isolates possessing the H278Y mutation were

found at lower frequencies in 2013-2015 compared to 2011 (Fig. 1.3A). From 2013-2015, the frequency of H134R-mutant *A. solani* isolates was significantly higher compared to 2011, comprising over 40% of isolates in a number of states, particularly in the Midwest (Fig. 1.3C). The frequencies of both H133R- and D123E-mutant isolates increased from 2013-2015 (Fig. 1.3D; Fig. 1.4A). Interestingly, in 2011, D123E-mutant isolates comprised 1.5% of isolates collected but consisted of 5%, 10%, and 12% of isolates collected in 2013, 2014, and 2015, respectively (Fig. 1.4A).

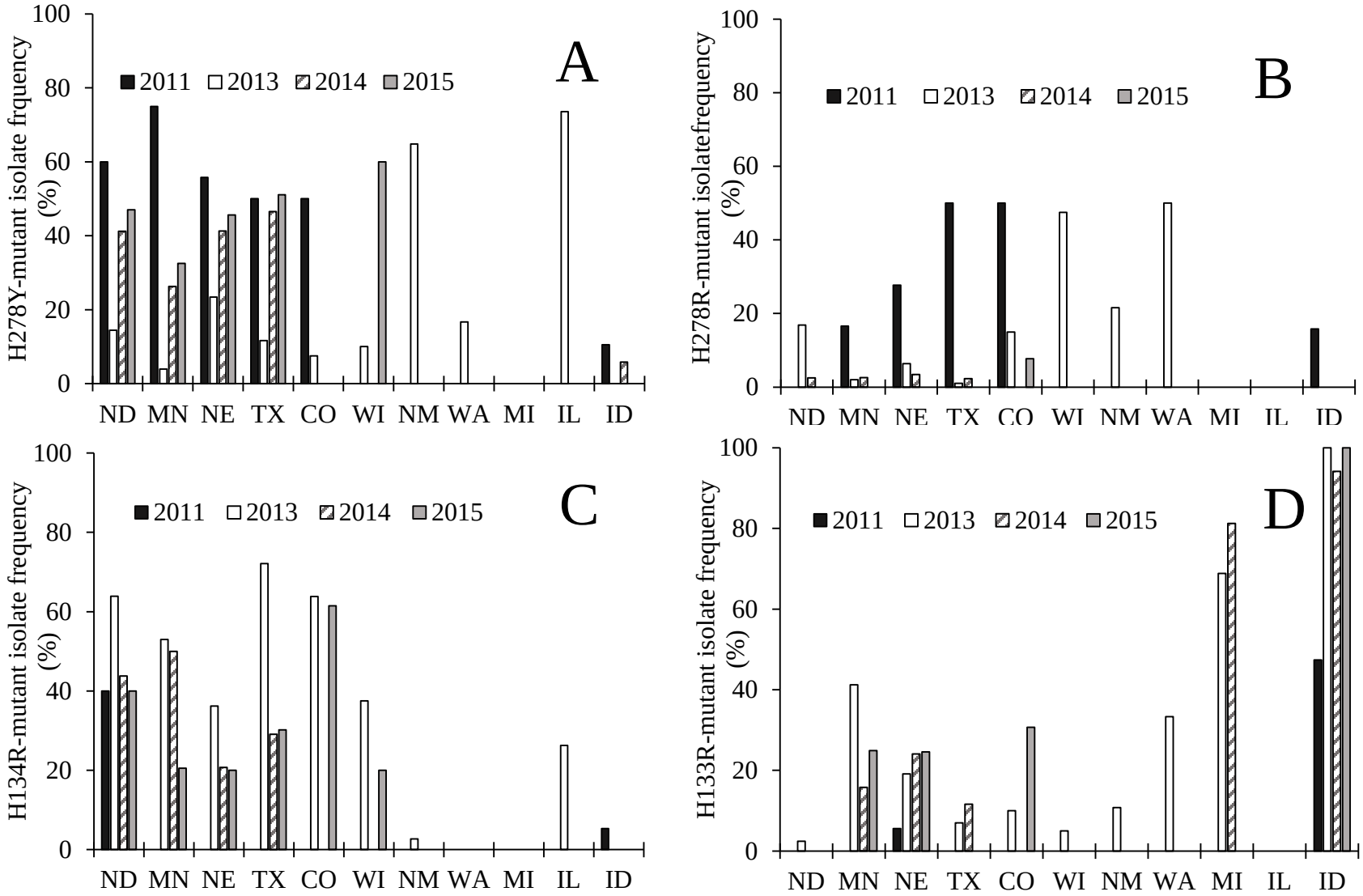

Fig. 1.3. Frequency of *Alternaria solani* isolates in each state possessing **A**, the H278Y mutation on *AsSdhB* gene; **B**, the H278R mutation on *AsSdhB* gene; **C**, the H134R mutation on the *AsSdhC* gene; and **D**, the H133R mutation on the *AsSdhD* gene each year of collection.

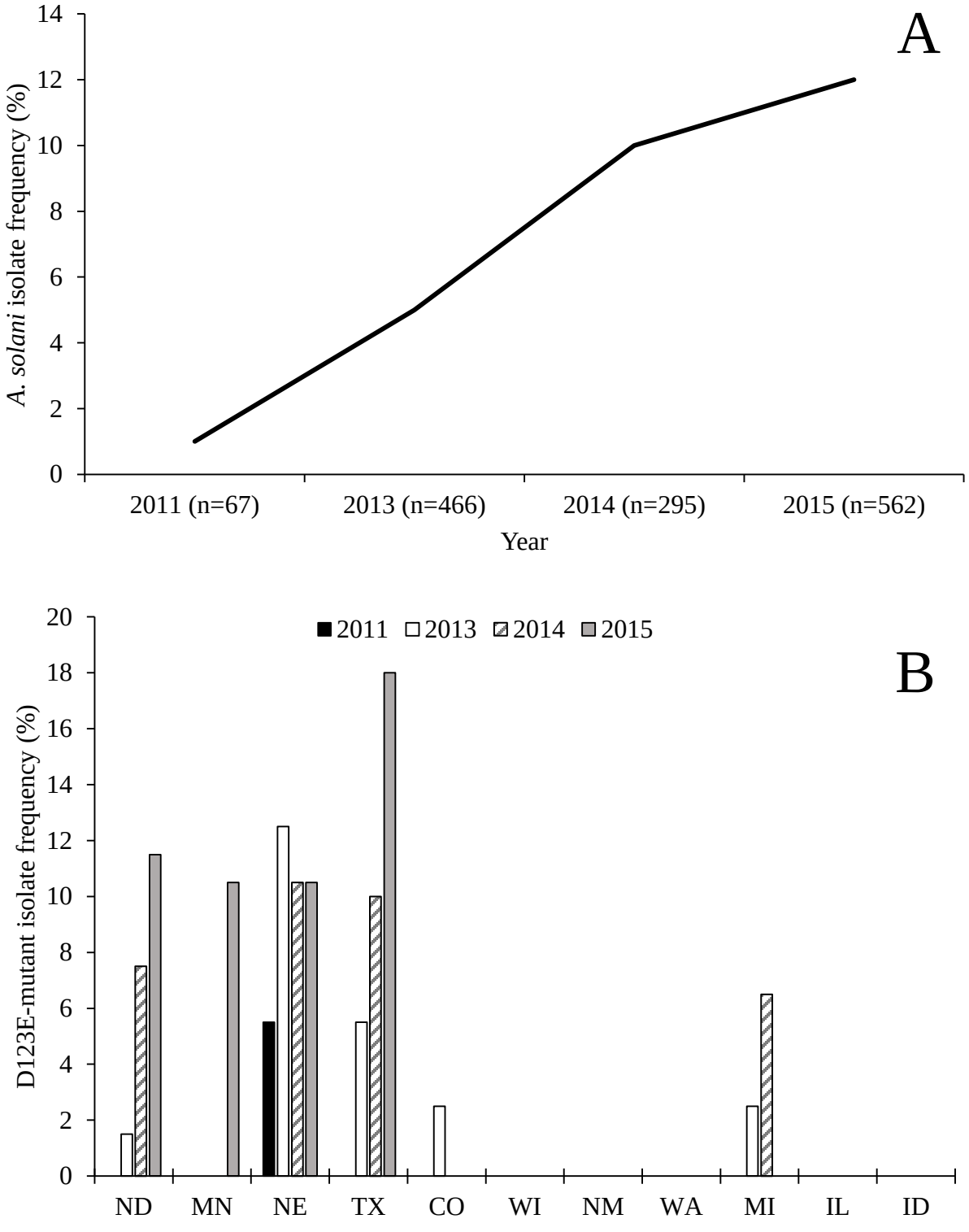

Fig. 1.4. A, Temporal and **B**, spatial frequency of *Alternaria solani* isolates possessing the D123E mutation on the *AsSdhD* gene conferring resistance to succinate dehydrogenase inhibiting (SDHI) fungicides.

Determination of spatial frequency distribution of mutations conferring QoI and SDHI resistance in *A. solani*. Maximum likelihood ANOVA from log-linear analysis also determined that state of collection had a significant effect (P=0.05) on the frequencies of mutations conferring SDHI and QoI resistance (Table 1.2). The state collected from was shown to have a significant effect on the frequency of the F129L-mutant *A. solani* isolates ($\chi^2 = 597.57$, P = <.0001) (Table 1.2). State, or location of the isolate sample, was also shown to have significant effect on the frequency of *A. solani* isolates possessing mutations on the *AsSdhB,* *AsSdhC,* and *AsSdhD* genes. This means that as a main effect, state was determined to be a significant factor for the frequency of the five mutations associated with SDHI resistance in *A. solani* isolates.

Analysis of maximum likelihood estimates from log-linear analysis for the main effects of year and state on the frequency of mutations conferring QoI and SDHI resistance in *A. solani* determined that the probabilities of collecting mutant isolates were significantly different among states (Table 1.4). It was determined that probability of collecting *A. solani* isolates with the F129L mutation was significantly higher in the states of North Dakota, Minnesota, Nebraska, Texas, Washington, Michigan, and Idaho. The probability of collecting *A. solani* isolates possessing the H278Y mutation was the highest in the states of Minnesota, North Dakota, and Wisconsin (P = 0.0227, 0.0098, and 0.0004, respectively) (Table 1.4). Although identified in only a small percentage of total isolates collected in 2014 and 2015, the probability of recovering H278R-mutant *A. solani* isolates is the greatest in Minnesota and Wisconsin (P = <.0001, and 0.0092, respectively). The probability of identifying H134R-mutant isolates in the states of North Dakota, Minnesota, Colorado, and Texas are significantly higher than other states (P = <.0001) (Table 1.4). The states of Minnesota and Nebraska were determined to have significantly higher

probabilities of recovering H133R-mutant isolates compared to other states. *Alternaria solani* isolates possessing the D123E mutation on the *AsSdhD* gene had a significantly higher probability of being collected in Texas (P = 0.0034) compared to other states (Table 1.4). The overall percentage of isolates collected possessing the D123E mutation significantly increased over the years of the survey and were recovered at higher frequencies in North Dakota, Minnesota, Michigan, Colorado, Nebraska, and Texas from 2013-2015 (Fig 1.4A; B).

Relative to 2011, F129L-mutant *A. solani* isolates were collected at a greater frequency and comprised a high percentage of isolates recovered across all 11 states from 2013-2015. Nearly 100% of the isolates in North Dakota, Minnesota, Texas, Colorado, and Idaho possessed the F129L mutation conveying resistance to QoI fungicides (Fig. 1.5). Mutant isolates possessing the H278Y mutation on the *AsSdhB* gene were collected at lower frequencies in North Dakota, Minnesota, Nebraska, and Colorado compared to 2011, but still comprised a large percentage of isolates collected across those states in 2015, as well as in states not previously collected from in 2011 including Wisconsin, New Mexico, Washington, and Illinois (Fig. 1.3A). Although identified in a large percentage of isolates in Texas, Colorado, and Idaho in 2011, *A. solani* isolates possessing the H278R mutation on the *AsSdhB* gene decreased in frequency across all states from 2013-2015. In 2015, H278R-mutant isolates were identified as a small percentage of isolates collected from Colorado and were not detected in isolates recovered from any other state (Fig. 1.3B). The frequency of *A. solani* isolates possessing the H134R mutation increased across a number of states relative to 2011, comprising a high percentage of isolates collected across several states including North Dakota, Minnesota, Nebraska, Texas, Colorado, and Wisconsin in 2013-15 (Fig. 1.3C). In 2011, only two states had *A. solani* isolates possessing the H133R mutation on the *AsSdhD* gene. In the current study, the H133R mutation was detected in 10 out

of 11 states and comprised nearly 100% of isolates collected from Idaho in 2013-2015 (Fig. 1.3D).

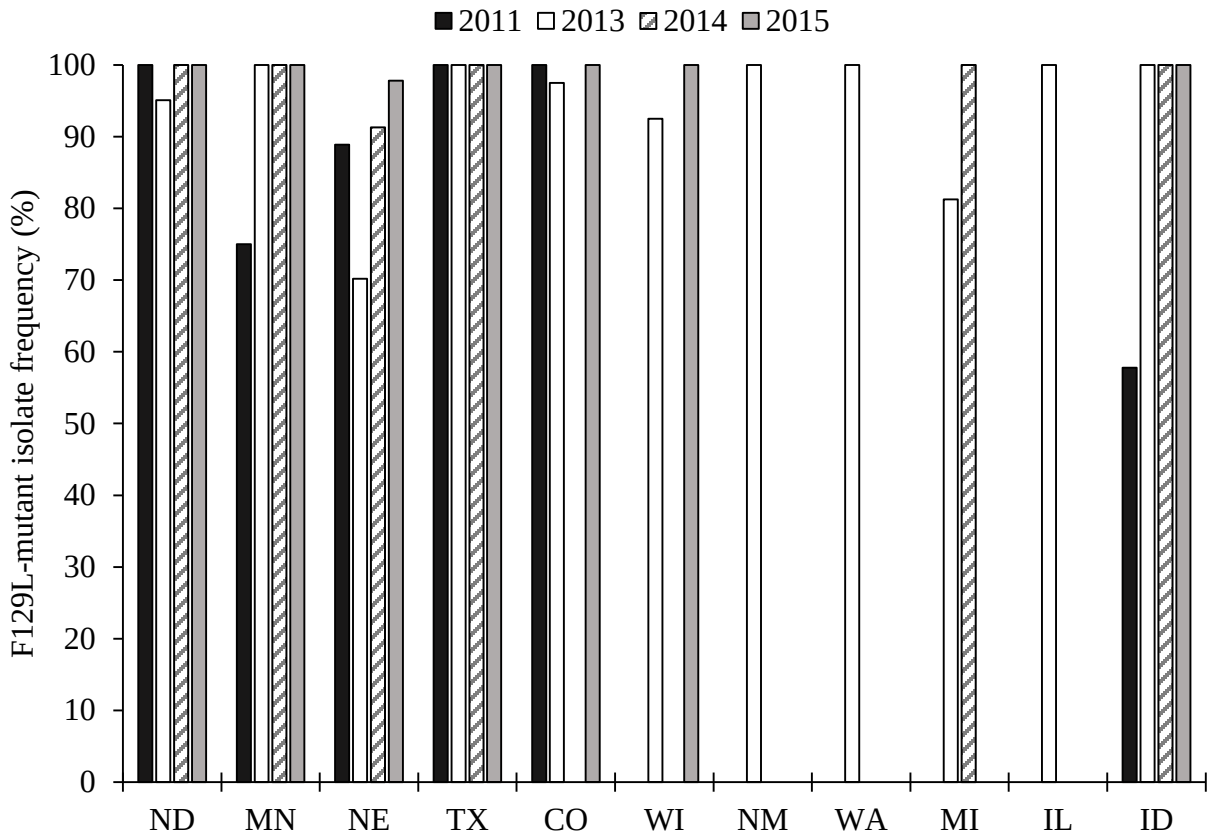

Fig. 1.5. Frequency of *Alternaria solani* isolates in each state possessing the F129L mutation conferring resistance to quinone outside inhibiting (QoI) fungicides each year of collection.

Prevalence of genotypes I and II in a diverse population of *A. solani* isolates collected throughout the United States. It was determined that the percentage of genotype II *A. solani* isolates was significantly higher than genotype I across all 11 states from which isolates were collected and across all years. In fact, over 99% of all isolates collected throughout the spatial-temporal survey were genotype II, with only small percentage of *A. solani* isolates, less than 1% total, characterized as genotype I (data not shown).

Discussion

The research reported here demonstrates that mutations associated with SDHI and QoI resistance are prevalent in many potato production regions of the United States and that there is significant spatial and temporal variability in the distribution of these mutations. This is the first report of the frequencies of these mutations in a spatially and temporally diverse collection of *A. solani* isolates. Resistance to QoIs in many fungi such as *Alternaria alternata*, *Cercospora beticola*, *Botrytis cinerea*, *Ascochyta rabiei*, and *Venturia inaequalis* is the result of an amino acid substitution of glycine with alanine at position 143 in *cytb* gene (G143A), which conveys a high level of resistance (Ma et al. 2003; Bardas et al. 2010; Bolton et al. 2013; Delgado et al. 2013; Frederick et al. 2014). However, resistance to QoIs in *A. solani* has been attributed to the F129L mutation, or the substitution of phenylalanine with leucine at position 129 (Pasche et al. 2005) in the *cytb* gene. The F129L mutation conveys a moderate level of resistance to QoI fungicides such as azoxystrobin and pyraclostrobin, resulting in a 12- to 15-fold reduction in sensitivity (Pasche et al. 2005; Pasche and Gudmestad, 2008). Also within the *cytb* gene structure, two genotypes have been detected in *A. solani*, genotype I and genotype II (Leiminger et al. 2014). Sequence analysis revealed the occurance of two structurally different *cytb* genes, which differed in the presence (genotype I) or absence (genotype II) of an intron. This study was the first to determine the prevalence of each *A. solani* genotype in the United States. In German *A. solani* isolates, the F129L mutation was identified only in genotype II *A. solani*, but genotype I isolates were far more prevalent, occurring in 63% of the isolates collected (Leiminger et al. 2014). However, this study found a different scenario within the USA, where nearly 100% of isolates collected were characterized as genotype II, although the F129L mutation was found to occur in both genotypes. It has therefore been speculated that the F129L

substitution must have occurred independently in the USA and Germany and occurred at least twice in the USA as the F129L substitution was found in USA isolates of both genotypes (Leiminger et al. 2014).

Resistance to SDHI fungicides in *A. solani* was first detected when field isolates collected in 2009 and 2010 from Idaho were determined to be resistant to boscalid (Wharton et al. 2012). However, in that study, *A. solani* isolates were reported as either completely resistant or completely sensitive to boscalid and EC_{50} values were not reported. Further studies reported widespread resistance in *A. solani* to boscalid in a number of states (Gudmestad et al. 2013; Mallik et al. 2014) including Idaho (Fairchild et al. 2013). Another study identified four mutations in two *AsSdh* genes in isolates recovered in Idaho and determined to be resistant to boscalid in vitro (Miles et al. 2014). The H277R mutation in the *AsSdhB* gene, and the T28A, A47T, and H133R mutations in the *AsSdhD* gene were detected in seven out of a total of only 11 *A. solani* isolates evaluated in that study. Furthermore, many of these seven isolates were determined to possess more than one of these four mutations (Miles et al. 2014). Most studies suggest that mutations associated with SDHI resistance independently confer resistance (Avenot et al. 2009; Avenot and Michailides, 2010), as was found in a previous study in our laboratory which presented corresponding EC_{50} values proving reduced sensitivity to high levels of resistance to boscalid and penthiopyrad for each of the five mutations characterized (Mallik et al. 2014).

The previous study by our research group characterized a total of 67 *A. solani* isolates for the presence of mutations associated with SDHI resistance (Mallik et al. 2014). Mutations on the *AsSdhB* gene were not only the most commonly detected, but were also demonstrated to be generally distributed among the six states of Colorado, Idaho, Minnesota, North Dakota,

Nebraska, and Texas. This was not determined to be the case with mutations on the *AsSdhC* and *AsSdhD* genes as isolates possessing these mutations were found to be more region specific (Mallik et al. 2014). Additionally, only a single isolate out of the 67 total that were characterized in the previous study was found to possess the D123E mutation on the *AsSdhD* gene associated with high boscalid and penthiopyrad resistance (Mallik et al. 2014). Compared to the initial survey conducted in 2011 by our lab, the results of this study using *A. solani* isolates collected from 2013 through 2015 suggests significant temporal changes in the frequencies of each mutation. Mutations conferring SDHI resistance in the *AsSdhB* gene were collected at a lower frequency, while mutations on the *AsSdhC* and *AsSdhD* gene were collected at a higher frequency from 2013 to 2015 compared to the previous study. However, from 2013 to 2015, the H278Y mutation was detected in isolates collected from all states except Michigan and was still identified in more than 20% of *A. solani* isolates collected from states across the Midwest including North Dakota, Minnesota, Nebraska, Texas, and Colorado. The H278R mutation was identified in significantly lower frequencies in 2013 through 2015 compared to 2011, comprising less than 1% of the 562 isolates collected in 2015 compared to 20% of the 67 isolates characterized in 2011. The current survey in this study identified the H134R mutation on the *AsSdhC* gene to be the most prevalent, with 36% of *A. solani* isolates collected possessing this mutation.

This study also suggests significant spatial variability in the mutations associated with SDHI resistance in *A. solani*. It was found that the main effects of collection year and state collected from had significant effect on the frequency of *A. solani* isolates possessing mutations conferring resistance to QoI and SDHI fungicides. Specifically, the combined effect of year and state also had significant effect on the frequency of *A. solani* isolates possessing mutations,

except those possessing the D123E mutation, associated with QoI and SDHI resistance. *A. solani* isolates possessing mutations on the *AsSdhB* gene were shown to be more prevalent in the states of North Dakota, Minnesota, and Wisconsin. However, *A.solani* isolates possessing the H134R mutation on the *AsSdhC* gene were shown to have a higher probability of being collected in Colorado and Texas, in addition to North Dakota and Minnesota. H133R-mutant isolates were shown to be more prevalent in Minnesota and Nebraska, and therefore, have a higher probability of being collected in those states relative to other locations around the United States. Finally, isolates possessing the D123E mutation on the *AsSdhD* gene are being detected at an increasingly higher frequency relative to isolates possessing other mutations associated with SDHI resistance development, and have the highest probability of being recovered in the state of Texas. Multiple states across the United States were shown to have a populations dominated by *A. solani* isolates resistant to QoI fungicides by virtue of possessing the F129L mutation and 100% of isolates collected from several of the 11 states included in this study were shown to possess the mutation. This suggests that an increasing percentage of isolates from *A. solani* populations across the country possess the F129L mutation relative to previous studies (Mallik et al. 2014; Pasche and Gudmestad, 2008).

There are several possible reasons for our observed shift in frequencies of mutations conferring SDHI and QoI resistance in *A. solani*. This study involved the sampling of infected foliar and tuber tissue from 11 states. However, not all 11 states were sampled in each year and in some states, a large number of isolates were collected and in other states, a smaller number of isolates were collected. A previous study identified aggregation in the spatial distribution patterns of SNPs related to fungicide resistance in *B. cinerea*, which has important implications for sample size and methods (Van der Heyden et al. 2014) and suggests that if a pathogen

population is aggregated, large sample sizes are required. Another possible reason for the shifts in spatial and temporal frequency of mutations presented here is the various levels resistance to foliar fungicide chemistries of the SDHIs and QoIs conveyed by the various identified mutations. For example, the H134R mutation in the *AsSdhC* gene was observed most commonly in *A. solani* isolates with high levels of boscalid and penthiopyrad resistance. Similarly, the H133R and D123E mutations in the *AsSdhD* gene were also observed in isolates with high levels of boscalid resistance. However, isolates possessing the H278Y or H278R mutation in the *AsSdhB* gene were associated with moderate levels of resistance to boscalid and penthiopyrad. The H278Y mutation was shown to be associated with high levels of boscalid resistance but only a moderate level of resistance to penthiopyrad, and the H278R mutation was shown to confer only moderate levels of resistance to both SDHI chemistries (Gudmestad et al. 2013; Mallik et al. 2014). Comparatively, these mutations confer differing levels of resistance to SDHI fungicides and mutations associated with resistance on the *AsSdhC* and *AsSdhD* genes consistently confer higher levels of resistance to boscalid and penthiopyrad than mutations on the *AsSdhB* gene. Mutations identified conferring resistance to SDHI fungicides in *B. cinerea* were also shown to have differential effects as different mutations had different sensitivities between different SDHI chemistries (Veloukas et al. 2011, 2013). Therefore, the results of this study may be due to selection for *A. solani* isolates possessing mutations conferring a higher level of resistance. Furthermore, since nearly 100% of *A. solani* isolates identified as possessing a mutation conferring SDHI resistance also possess the F129L mutation conveying QoI resistance, selection for multiple chemical classes is present in *A. solani* populations across the United States. This phenomena of selection for resistance to multiple chemical classes at once, termed "selection by association," is based on the assumption that an isolate with resistance to multiple chemical

classes would be selected by the application of any one those chemical classes (Hu et al. 2016). This indirect selection, or selection by association, could be a reason why *A. solani* isolates resistant to both SDHI and QoI fungicides are predominant across the country. It is also technically possible that fungicide-induced mutagenesis could be accelerating the genetic changes in field populations in *A. solani* and why we are seeing an increase in the occurrence of these mutations associated with fungicide resistance within and between populations. However, this was previously investigated in *Monilinia fructicola* where it was found that fungicide-induced genetic changes may not readily occur under field conditions (Dowling et al. 2016).

The most obvious reason for the shift in spatial and temporal frequency of mutations in *A. solani* associated with SDHI and QoI resistance, and a critical aspect in SDHI resistance development, is the existence of possible fitness penalties of target mutations (Sierotzki and Scalliet, 2013). Previous studies with *A. solani* could not identify any fitness penalties associated with QoI resistance (Pasche and Gudmestad, 2008). The existence of multiple mutations conferring SDHI resistance in *A. solani* and the differing levels of reduced sensitivity conferred by them does warrant further study comparing pathogenic fitness of wild-type vs. mutant isolates. The existence of any significant fitness penalties in fungicide-resistant isolates of any pathogen can predict the efficacy of fungicide resistance management tactics, such as tank-mixing, in delaying or preventing the development of further resistance issues (Mikaberidze et al. 2014). The assessment of fitness costs of fungicide resistance mutations is crucial to determine if fungicide mixtures select for resistance. Some studies have identified fitness costs in fungicide resistance pathogen populations in both laboratory and field settings (Iacomi-Vasilescu et al. 2008; Karaoglanidis et al. 2001). Specifically, a recent study of fitness of *B. cinerea* field isolates with dual SDHI and QoI resistance determined that isolates with mutations in the

BcSdhB gene may be adversely affected and that sensitive isolates dominated in the absence of fungicide selection pressure in competition experiments (Veloukas et al. 2014). Most evidence, however, suggests that fitness penalties associated with fungicide resistance in other pathogens are either low (Billard et al. 2012; Kim and Xiao, 2011) or completely absent (Corio-Costet et al. 2010). In a previous study, no significant fitness penalties were identified in *A. solani* isolates possessing the F129L mutation conferring QoI resistance (Pasche et al. 2008). Further monitoring of mutation prevalence in *A. solani* populations conferring fungicide resistance will be important as foliar fungicide application regimes change or as new SDHI chemistries, such as solatenol and adepidyn (Syngenta®), are registered for use in potato.

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CHAPTER 2. POTENTIAL IMPACT OF FLUOPYRAM ON THE FREQUENCY OF THE D123E MUTATION IN *ALTERNARIA SOLANI*

Abstract

Succinate dehydrogenase inhibiting (SDHI) fungicides have been widely applied in commercial potato (*Solanum tuberosum* L.) fields for the control of early blight, caused by *Alternaria solani* Sorauer. Five point mutations on three *AsSdh* genes in *A. solani* have been identified as conferring resistance to SDHI fungicides. Recent work in our laboratory determined that *A. solani* isolates possessing the D123E mutation, or the substitution of aspartic acid for glutamic acid at position 123 in the *AsSdhD* gene, were collected at significantly higher frequencies throughout a three year survey. A total of 118 *A. solani* isolates with the D123E mutation were evaluated in vitro for boscalid and fluopyram sensitivity and their EC_{50} values, ranging from 0.2 to 3 µg/ml, were sensitive and only slightly higher than those of baseline isolates, ranging from 0.1 to 0.6 µg/ml. Fifteen isolates characterized as being SDHI-sensitive or -resistant, including five isolates with the D123E mutation, were evaluated in vivo for percentage disease control to boscalid and fluopyram. Area under the dose response curve (AUDRC) values for boscalid and fluopyram were significantly lower for all five D123E-mutant isolates, demonstrating reduced disease control in vivo. In field trials, the frequency of *A. solani* isolates with the D123E mutation recovered from treatments receiving an in-furrow application of fluopyram ranged from 5 to 37%, which was significantly higher compared to treatments receiving foliar applications of standard protectants, ranging from 0 to 2.5%. Results suggest *A. solani* isolates possessing the D123E mutation have a selective advantage under the application of fluopyram compared to SDHI-sensitive isolates, as well as isolates possessing other mutations conferring SDHI resistance. These data illustrate the importance of implementing fungicide

resistance management strategies and cautions the labeling of fluopyram for in-furrow applications that target other pathogens of potato.

Introduction

Alternaria solani Sorauer, which causes early blight of potato (*Solanum tuberosum* L.), creates characteristic dark brown to black lesions with a concentric ring pattern on senescing leaves. Early blight is a ubiquitous disease and particularly problematic on potatoes grown under irrigation and during periods of heavy dew (Rotem, 1994). In addition to inducing premature defoliation of infected potato plants, resulting in tuber yield reduction, *A. solani* can also cause a type of dry rot on tubers which decreases quality (Nnodu et al. 1982). The majority of commercially acceptable potato cultivars are susceptible to early blight; therefore, the frequent application of foliar fungicides is the primary management tactic to achieve sufficient control (Gudmestad et al. 2013; Yellareddygari et al. 2016).

Standard protectants, such as chlorothalonil and mancozeb, are perhaps the most frequently applied fungicides for early blight management, but are insufficient under high inoculum pressure and conducive environmental conditions (Fonseka and Gudmestad, 2016; Yellareddygari et al. 2016). As a result, the application of locally systemic and translaminar fungicides, such as quinone outside inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs), may be necessary to slow the development of an early blight epidemic. QoI fungicides were used successfully following their introduction in 1999, but resistance was detected in North Dakota and Nebraska by 2001 (Pasche et al. 2004, 2005), and determined to prevalent throughout much of the United States by 2006 (Pasche and Gudmestad, 2008). While resistance to QoI fungicides in many host-pathogen systems results from the amino acid substitution of glycine with alanine at position 143 (G143A) in the cytochrome b (*cytb*) gene, reduced

sensitivity in *A. solani* has been attributed to the F129L mutation, or the substitution of phenylalanine with leucine at position 129 in *cytb* (Pasche et al. 2005). The F129L mutation in *A. solani* isolates results in a 12- to 15-fold reduction in sensitivity to azoxystrobin.

The target site of SDHI fungicides is mitochondrial complex II at either the succinate ubiquinone reductase or succinate dehydrogenase complex, which is a functional part of the mitochondrial electron transport chain (Avenot and Michailides, 2010; Sierotzki and Scalliet, 2013). The SDHI fungicide boscalid was registered in the United States for use on potato in 2005 and initially provided excellent early blight control (Fairchild et al. 2013; Gudmestad et al. 2013). Unfortunately, resistance to bosalid has developed in a number of different host-pathogen systems including *A. alternata* of pistachio (Avenot and Michailides, 2007), *Botrytis cinerea* in several crops (Bardas et al. 2010; Leroch et al. 2011; Myresiotis et al. 2008), *Corynespora cassiicola* of cucumber (Miyamoto et al. 2009; Ishii et al. 2011), *Didymella bryoniae* of cucurbits (Avenot et al. 2012), *Monilinia fructicola* of peach (Amiri et al. 2010), and *Venturia inaequalis* of apple (Toffolatti et al. 2016). Boscalid resistance was first detected in *A. solani* in field isolates collected in 2009 and 2010 from Idaho (Wharton et al. 2012) and found to be prevalent throughout a number of states including North Dakota, Minnesota, Nebraska, and Texas in 2011 (Gudmestad et al. 2013).

Five known point mutations on three *AsSdh* genes have been characterized to independently confer resistance to SDHI fungicides (Mallik et al. 2014). Point mutations on the *AsSdhB* gene leading to an exchange from histidine to tyrosine (H278Y) or arginine (H278R), respectively, at codon 278 are associated with moderate to high levels of resistance to boscalid and penthiopyrad. A point mutation in the *AsSdhC* gene leading to an exchange from histidine to arginine (H134R) at codon 134 confers very high boscalid and penthiopyrad resistance. Two

additional point mutations on the *AsSdhD* gene leading to exchanges from histidine to arginine (H133R) at codon 133, and aspartic acid to glutamic acid (D123E) at codon 123, are also associated with high levels of resistance to boscalid and penthiopyrad (Mallik et al. 2014).

The SDHI fungicide fluopyram was registered for use on potato in 2012 and become a reliable alternative to QoI fungicides as well as other SDHIs. A complete lack of cross resistance between boscalid and fluopyram has been demonstrated among *A. solani* isolates that have developed resistance to boscalid (Fairchild et al. 2013; Gudmestad et al. 2013). While the lack of cross resistance between fluopyram and boscalid in other fungi has been speculated to be attributed to a higher intrinsic activity of fluopyram compared to boscalid, there has been no evidence to suggest differences in intrinsic activity of these two chemistries in a baseline population of *A. solani* (Gudmestad et al. 2013). Therefore, it is more likely that fluopyram binds at a different site on the iron sulfur protein or elsewhere in complex II than boscalid and is unaffected by the same mutations (Avenot and Michailides, 2010; Gudmestad et al. 2013). However, the increased and widespread application of fluopyram in commercial potato fields will place significant selection pressure on the early blight pathogen, and in turn, may lead to the development of resistance. Given the ability of *A. solani* to develop resistance to SDHI, QoI, and anilino-pyrimidine (AP) fungicides, continued monitoring of further resistance development is crucial (Pasche et al. 2004; Fairchild et al. 2013; Gudmestad et al. 2013; Fonseka and Gudmestad, 2016).

In our previous study, *A. solani* isolates possessing the D123E mutation comprised 1.5% of isolates collected in 2011 (Mallik et al. 2014). In a more recent survey from 2013 through 2015, D123E-mutant isolates were recovered at significantly higher frequencies of 5 to 12% of isolates collected (Bauske et al. Plant Disease in review). However, little is known about the

sensitivity of *A. solani* isolates possessing the D123E mutation to SDHI fungicides in vitro or in vivo. As a result, it becomes critical to determine the potential causes associated with the increase in frequency of D123E-mutant *A. solani* isolates and evaluate the sensitivity of these isolates under the application of SDHI fungicides. The specific objectives of the current study were to (i) determine in vitro sensitivity of *A. solani* isolates possessing the D123E mutation to the SDHI fungicides boscalid and fluopyram; (ii) determine the disease control of D123E-mutant *A. solani* isolates under the application of boscalid and fluopyram in vivo; and (iii) determine the effect of the D123E mutation on early blight disease control under field conditions.

Materials and Methods

***A. solani* isolate collection and storage.** Infected leaf samples were submitted to our laboratory during the 2013, 2014, and 2015 potato growing seasons from numerous potato producing regions across the United States, including North Dakota, Minnesota, Nebraska, New Mexico, Texas, Michigan, Illinois, Wisconsin, Idaho, Washington, and Colorado (Table 2.1). A total of 118 *A. solani* isolates with the D123E mutation were evaluated during the course of these studies. Approximately 15 to 20 infected leaves were randomly collected from each potato field, stored with a damp paper towel in a plastic bag, and mailed directly to our laboratory. Three to five infected leaflets from each field were stored in a herbarium press for preservation of the original collection. Early blight lesions from leaf samples were transferred to 1.5% agar media and were incubated at room temperature (22 ± 2°C) for 3 to 4 days until conidia were produced (Holm et al. 2003). A single conidium was transferred to a solid clarified V8 media (CV8) (Campbell's V8 juice, 100 ml; $CaCO_3$, 1.5g; agar, 15g; and distilled water, 900 ml) amended with 50 mg/ml ampicillin. Cultures were incubated under continuous fluorescent light for 7 to 10

days and examined for the presence of *A. solani* conidia under a stereomicroscope (Pasche et al. 2004). For long-term cryogenic storage, 4-mm diameter plugs of media with fungal mycelia and conidia were cut using a sterilized cork borer, and the plugs were placed in screw-top centrifudge tubes (Fonseka and Gudmestad, 2016). The loosely capped tubes were placed in closed container with silica gel for 2 to 3 days to remove moisture from the media and capped, sealed with Parafilm, and stored at -80°C in an ultrafreezer.

Table 2.1. Origin and frequency by state of D123E-mutant *Alternaria solani* isolates collected from 2011 through 2015 evaluated in vitro.

States	Year	N[z]
Colorado	2013	2
Michigan	2013	4
	2014	1
Minnesota	2015	19
Nebraska	2013	6
	2014	6
	2015	14
North Dakota	2013	15
	2014	6
	2015	8
Texas	2013	5
	2014	9
	2015	23
Total	2013	32
	2014	22
	2015	64
Total	-	118

[z] Number of Alternaria solani isolates possessing the D123E mutation evaluated in vitro.

DNA extraction. DNA was extracted from all isolates using a modified cetyltrimethylammonium bromide (CTAB) method (Mallik et al. 2014; Stewart and Via, 1999). First, mycelia and spores were scraped from a 7-day old culture of *A. solani* into an autoclaved mortar and ground to fine powder with liquid nitrogen. Approximately 100mg of powder was transferred immediately into a tube of lysing matrix A (MP Biomedicals LLC, OH) consisting of

750 µl of Carlson lysis buffer (100 mM Tris HC1 [pH 9.5], 2% CTAB, 1.4 M NaCl, 1% PEG 8000, and 20 mM EDTA) supplemented with 2% β-mercaptoethanol. The tube was placed in a FastPrep instrument (MP Biomedicals) and subjected to agitation at a speed of 6.00 m/s for 40 s to facilitate the homogenization of the mycelia and spore mixture. The sample was incubated in the tube at 75°C for 40 min with inversions at intervals of 10 to 15 min followed by a centrifugation at a speed of 14,000 × g for 10 min (Mallik et al. 2014). The supernatant was removed and placed immediately into a new tube. Nucleic acids were extracted in the aqueous phase by adding an equal volume of phenol/chloroform/isoamyl alcohol, 25:24:1(vol/vol) (Sigma-Aldrich, St. Louis, MO). Genomic DNA was then precipitated with an equal volume of isopropanol and washed with 95% ethanol. Finally, genomic DNA was reconstituted in a glass-distilled RNA-ase-DNAase-free water (Teknova Inc., Hollister, CA) at a final concentration of 10 ng μl^{-1} and RNA-ase (0.2 µg) (Qiagen Inc. Valencia, CA) was added.

Detection of the D123E mutation in *A. solani* isolates. A polymerase chain reaction (PCR) was performed in a thermal cycler DNA engine, with an initial preheat step of 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min and performed using 23-µl volume consisting of 20 ng of DNA, 1.5 mM $MgCl^2$, 0.5 mM dNTP, 5µM each primer, and 1 U of Go Taq polymerase (Promega Corp.)(Mallik et al. 2014). Isolates that yielded a 127-bp amplification product with this reaction possess the D123E mutation. All amplified products were separated by gel electrophoresis in 1.2% agarose gel (Mallik et al. 2014).

In vitro sensitivity of D123E-mutant *A. solani* isolates to SDHI fungicides. In vitro sensitivity was evaluated via conidial germination assays conducted as previously described (Gudmestad et al. 2013) using 7- to 14-day-old cultures of *A. solani* maintained under continuous

fluorescent light at 22 ± 2°C. A glass rod was used to free conidia from the surface of CV8 media using sterile, distilled water under aseptic conditions. The conidial concentration of the suspension was adjusted to 2×10^4 conidia/ml, 150 ml was added to the surface of fungicide amended media. Media containing 2% laboratory grade agar (A360-500 Fisher Scientific, Pittsburgh, PA) were amended with technical grade boscalid (99% active ingredient [a.i.]; BASF Corporation, Research Triangle Park, NC) or fluopyram (98.78% a.i.; Bayer CropScience, Monheim am Rhein, Germany) dissolved in acetone (approximately 10 mg of a fungicide per ml) and diluted in deionized water to reach final concentrations of 0, 0.01, 0.1, 1, 10, and 100 mg/ml. The final concentration of acetone in the media was 0.1% by volume. Salicylhydroxamic acid (SHAM) was dissolved in methanol and added to the media to reach a final concentration of 100 mg/ml to be consistent with previous SDHI studies (Gudmestad et al. 2013; Pasche et al. 2005).

For all in vitro conidial germination assays, petri plates were incubated at 21°C under continuous light for 4 h prior to evaluation. Following incubation, 50 conidia were examined for the development of one germ tube as long as the conidium, or multiple germ tubes developing from one conidium, using a microscope at ×100 magnification (Gudmestad et al. 2013; Pasche et al. 2005). All experiments were performed twice with two replicates for each fungicide concentration. In total, 118 *A. solani* isolates possessing the D123E mutation were evaluated for in vitro sensitivity to boscalid and fluopyram in 20 trials, with 9 to 13 isolates included in each trial. Control isolates 13-1, a wild-type *A. solani* isolate, and 526-3, a QoI-resistant *A. solani* isolate, were included in each trial as internal controls. In addition, individual *A. solani* isolates possessing mutations H133R or H134R with EC_{50} values <5.0 (Mallik et al. 2014) were also

included. These five isolates were re-assayed in vitro, in two separate trials as described above, to insure that the reported EC_{50} values were precise.

Disease control of D123E-mutant *A. solani* isolates under the application of boscalid and fluopyram in vivo. Fifteen *A. solani* isolates were selected for in vivo evaluation based on in vitro boscalid and fluopyram sensitivity (Table 2.2), including five D123E-mutant isolates and five SDHI-sensitive isolates possessing none of the five known point mutations associated with SDHI resistance. The remaining five *A. solani* isolates possessed other mutations conferring SDHI resistance, but a low level of insensitivity to boscalid and fluopyram (EC_{50} values <.05). All isolates selected for in vivo experiments had very similar EC_{50} values to boscalid and fluopyram in vitro. The fitness of *A. solani* isolates under the application of these two SDHI fungicides was evaluated under greenhouse conditions as previously described (Gudmestad et al. 2013; Fonseka and Gudmestad, 2016; Pasche et al. 2004; 2005).

Table 2.2. Origin, mutation characterization, and in vitro boscalid and fluopyram sensitivity of *Alternaria solani* isolates chosen for in vivo experiment.

SDHI Mutation[x]	Isolate	State of Origin	F129L[y]	Boscalid EC_{50}[z](µg/ml)	Fluopyram EC_{50}[z](µg/ml)
SDHI-sensitive	1342-8	Nebraska	M	4.01	1.78
SDHI-sensitive	1331-6	Texas	M	3.12	1.20
SDHI-sensitive	1393-5	North Dakota	M	4.12	2.08
SDHI-sensitive	1393-8	North Dakota	WT	3.21	0.39
SDHI-sensitive	1486-11	North Dakota	WT	3.01	0.45
H133R	1172-8	Idaho	M	6.28	2.15
H133R	1176-3	Idaho	M	6.80	2.33
H134R	1174-9	Idaho	M	5.89	2.09
H134R	1231-9	North Dakota	M	3.34	1.55
H278R	1172-6	Idaho	M	5.01	0.11
D123E	1342-6	Nebraska	M	4.81	0.26
D123E	1332-2	Texas	M	7.34	1.74
D123E	1364-1	Colorado	M	4.54	0.38
D123E	1393-1	North Dakota	WT	4.21	2.46
D123E	1393-18	North Dakota	WT	6.18	3.13

[x] Isolate possesses none of the five mutations on three *AsSdh* genes conferring SDHI resistance.

[y] M = mutant isolate with the F129L mutation conferring QoI resistance. WT = wild type isolate.

[z] EC_{50} = the effective concentration at which the funal growth is inhibited by 50%.

Isolates of the early blight fungus were inoculated onto plants applied with different doses of each SDHI fungicide. The in vivo sensitivity assay was conducted as a 24-h preventative test in the greenhouse using tomato plants, cv. Orange Pixie VFT hybrid (Tomato Growers Supply Company, Fort Myers, FL) chosen for its susceptibility to early blight, compact size, and the fact that early blight affected leaves do not dehisce under severe disease conditions. Three tomato seeds were sown in each 10-cm^3 plastic pot containing Sunshine Mix LC1 (Sun Gro Horticulture Inc., Bellevue, WA), and following emergence, plants were thinned to obtain two uniformly sized plants per pot. When the first three leaves were fully expanded and plants had reached a height of 15 to 20 cm, plants were treated with a commercial formulation of

boscalid (70% a.i.; Endura™, BASF Corporation) or fluopyram (41.5% a.i.; Luna™, Bayer). Ten-fold fungicide concentrations of the active ingredient were applied to plants (0, 0.1, 1, 10, and 100 µg/ml) to obtain dose response curves. Fungicide was applied preventatively using a Generation III Research Sprayer (Devries Manufacturing, Hollandale, MN) starting with the lowest concentration and rinsed thoroughly with distilled water between fungicide chemistries.

For inoculation of *A. solani*, isolates maintained on solid CV8 media for 7 to 14 days under continuous fluorescent light at 22 ± 2°C were used to prepare a suspension of 2×10^5 conidia/ml sterile distilled water. Tomato plants were inoculated with 50 ml of conidial suspension/plant 24 h post-fungicide application using a Preval paint-spray gun (Preval Sprayer Division; Precision Valve Corporation, Yonkers, NY). Inoculated plants were kept in individual humidity chambers (Phytotronic Inc., Earth City, MO) for 24 h at >95% relative humidity and 22 ± 2°C and subsequently transferred to confinement chambers (plastic chambers with open ceilings) on greenhouse benches, to minimize inter-isolate interference, and maintained at 25 ± 2°C with daily application of water. Percentage disease severity was visually evaluated on three fully expanded true leaves on each of two plants/pot, one pot/replicate, and three replicates at 6, 9, and 12 days post-inoculation (DPI). The in vivo experiment was performed twice.

Effect of the D123E mutation on early blight disease control using SDHI fungicides in the field. Field trails were conducted in 2013 and 2015 evaluating the efficacy of fluopyram applied in-furrow at planting and as a foliar application during the growing season. Trials were performed under irrigated conditions as randomized complete block designs with four replications in Inkster, ND. Treatments were planted to cv. Ranger Russet, chosen for its susceptibility to early blight, in four row plots approximately 3.6 m wide and 9.0 m in length. The experiment consisted of eight separate treatments, including a non-treated control (Table

2.3). Two separate treatments consisted of foliar application of the standard protectants chlorothalonil and mancozeb, respectively, at 7-d intervals throughout the growing season. A treatment consisting of multiple applications of single-site foliar fungicides tank-mixed with standard protectants was also included. Three treatments including the in-furrow application of fluopyram alone, and together with two different rates of the biological control *Bacillus subtilis* (Serenade Soil™, Bayer CropScience), respectively, were also evaluated. An additional treatment consisting of the in-furrow application of the systemic insecticide imidacloprid (Admire Pro™, Bayer CropScience) applied along with fluopyram and Serenade Soil was included in the trial both years.

Table 2.3. Fungicide treatment, use rates, and application schedule of treatments evaluated in field trials conducted in 2013 and 2015 in northeastern North Dakota.

Treatment		Rate of a.i./ha[y]	Schedule[z]	Interval
1	Non-treated	-	-	-
2	Chlorothalonil	1.25 kg/ha	Full Season	7 day
3	Mancozeb	1.80 kg/ha	Full Season	7 day
4	Fenamidone +	0.25 kg/ha	1,3	7 day
	Sticker/Extender Adjuvant +	0.1% v/v +		
	Mancozeb	1.80 kg/ha		
	Chlorothalonil	0.75 kg/ha	2	
	Chlorothalonil	1.25 kg/ha	4,6,8-10	
	Fluopyram/Pyrimethanil +	0.09 kg/ha; 0.27 kg/ha	5	
	Mancozeb	1.80 kg/ha		
	Pyrimethanil +	0.30 kg/ha	7	
	Mancozeb	1.80 kg/ha		
5	Fluopyram	0.35 kg/ha	In-furrow	At Planting
6	Fluopyram +	0.35 kg/ha	In-furrow	At Planting
	Bacillus subtilis	4.68 l/ha		
7	Fluopyram +	0.35 kg/ha	In-furrow	At Planting
	Bacillus subtilis	9.36 l/ha		
8	Imidacloprid +	0.15 kg/ha	In-furrow	At Planting
	Fluopyram +	0.15 kg/ha		
	Bacillus subtilis	4.68 l/ha		

[y] l/ha = liter per hectare; kg/ha = kilogram per hectare.
[z] Week(s) fungicide was applied.

In-furrow applications were applied using a planter-mounted CO_2 sprayer with a single nozzle with 6501 tip directed at seed-piece. All foliar fungicides were applied with water volumes of 560 l ha^{-1} at 375 kPa to ensure adequate coverage. Percentage early blight severity was recorded in the center two rows at approximately 7-day intervals beginning in early to mid-July (about 60-70 days after planting) depending on disease pressure. Early blight disease severity evaluations, taken on a scale of 0-100% diseased leaf tissue, continued for 11 weeks, not surpassing 7 d after the final foliar fungicide application (Pasche and Gudmestad, 2008).

For inoculation of the treatments, four *A. solani* isolates, two containing the F129L mutation and two wild-type, were grown under constant fluorescent light for 2 weeks on CV8 medium at room temperature (22 ± 2°C). Distilled water was added to the cultures and conidia were dislodged with a glass rod and diluted in 0.25% gelatin to a concentration of 6.7×10^3 conidia/ml. This suspension was applied to the outside two rows of each four-row treatment at a rate of 104 ml/row on two days, approximately mid-July and early august, using custom ATV application equipment. Following the last foliar disease severity rating, approximately 10 infected leaves were sampled at random from all four replicates of each treatment, including the non-treated control, and placed in unsealed plastic bags inside a cooler and transported back to the laboratory. Early blight lesions on infected leaf tissue were then transferred to 1.5% agar media and isolations were made as previously described (Holm et al. 2003). DNA extractions and PCR was performed on each of the isolates collected as described above. Approximately 40 *A. solani* isolates from each treatment were collected each year and assayed to determine the prevalence of isolates possessing the D123E mutation among treatments.

Statistical analysis. To calculate EC_{50} values for in vitro experiments, the percentage reduction in germination relative to the non-fungicide-amended control was calculated, and

regressed against the $\log_{10}$ fungicide concentration. The concentration determined to reduce germination by half, compared to 0 µg/ml concentration, was extrapolated from the 50% intercept (EC_{50} value), using the Statistical Analysis System (SAS Institute Inc., Cary, NC). Approximate limits for a 95% confidence interval for the two internal controls were calculated as a measure of assay reproducibility (Wong and Wilcox, 2002). Trials in which the EC50 values of the internal controls were within the 95% confidence internal were included in analysis. Experiments were analyzed separately and an F-test used to assess homogeneity of variance among experiments.

In vivo experiments were arranged as split-plot randomized complete block designs with *A. solani* isolates as the main plot and fungicide concentrations as the split-plots. For each isolate, at all fungicide concentrations, disease severity data was transformed to percentage disease control, using the formula 1 – (% diseased tissue / % diseased tissue in non-treated plants) × 100 (Gudmestad et al. 2013; Fonseka and Gudmestad, 2016). Levene's test was conducted to determine homogeneity of variance between two independent experiments (Milliken and Johnson, 1992). Analysis of variance (ANOVA) was performed separately for isolate × fungicide group combination at each of the four fungicide concentrations (0.1, 1, 10, and 100 µg/ml) using SAS, and *t* tests were used on the combined data to determine differences at each fungicide concentration. Area under the dose response curve (AUDRC), or area under the disease progress curve across all doses of fungicide, was calculated to determine significant differences in disease control provided by boscalid and fluopyram in controlling SDHI-sensitive and SDHI-resistant *A. solani* isolates.

For field trials, percentage early blight severity, recorded as previously described above, was used to calculate the area under the disease progress curve (AUDPC) as follows (Shaner and Finney, 1977):

$$\text{AUDPC} = \sum_{i=1}^{n} [(\text{W}_{i+1} + \text{W}_i)/2][\text{t}_{i+1} - t_i],$$

Where W_i is the percentage foliar disease severity at the ith observation, t_i the time in days at the ith observation and n the total number of observations. The relative area under the disease progress curve (RAUDPC) was calculated for each treatment of the replicated trials from each year by dividing AUDPC values by the total area of the graph and analyzed using ANOVA (Proc GLM SAS version 9.3, Cary, NC). Fisher's protected LSD test (P = 0.05) was used to differentiate mean RAUDPC values (Pasche and Gudmestad, 2008). A χ^2 test was used to determine whether the frequency of D123E-mutant isolates collected differed among treatments in the field.

Results

Determination of in vitro sensitivity of *A. solani* isolates possessing the D123E mutation to the SDHI fungicides boscalid and fluopyram. Sensitivity to boscalid in *A. solani* isolates possessing the D123E mutation varied widely, ranging from 4.5 to >100 µg/ml (Fig. 2.1A), with a mean EC_{50} value of 60.7 µg/ml. This range was significantly higher compared to EC_{50} values of baseline isolates to boscalid, ranging from 0.1 to 1.5 µg/ml (Fig. 2.1A), with a mean EC_{50} value of 0.33 µg/ml. Sensitivity to fluopyram in *A. solani* isolates with the D123E mutation were similar to wild type baseline isolates, ranging from 0.2 to 3 µg/ml, with a mean EC_{50} value of 1.5 µg/ml compared to baseline isolates which ranged from 0.1 to 0.6 µg/ml (Fig. 2.1B), with a mean EC_{50} value of 0.31 µg/ml.

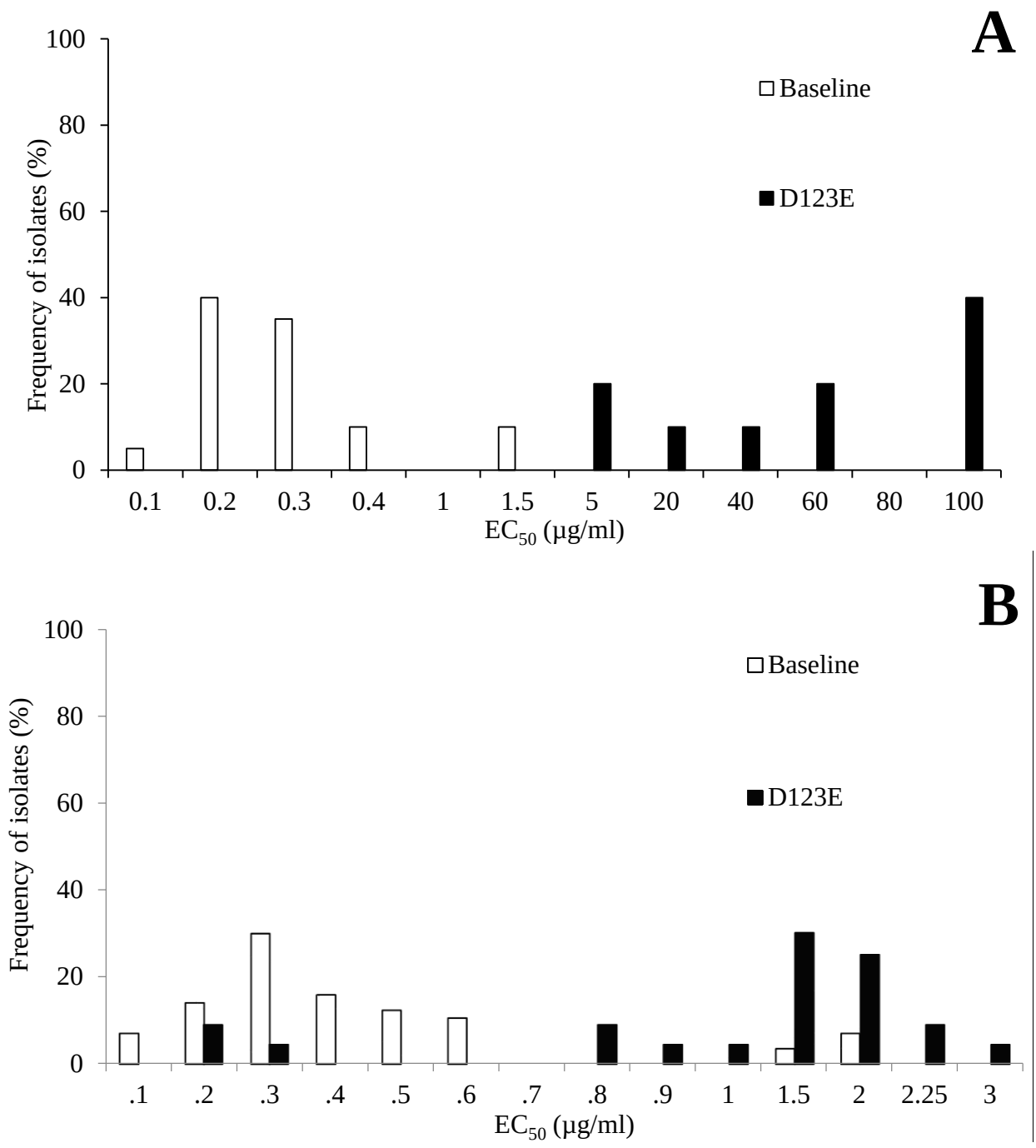

Fig. 2.1. Frequency distribution of sensitivity of 57 baseline *Alternaria solani* isolates and 118 D123E-mutant *Alternaria solani* isolates to **A**, boscalid and **B**, fluopyram, based on in vitro methods to determine effective concentration that inhibits spore germination by 50% compared to nontreated control (EC_{50} µg/ml). Sensitivities for baseline *Alternaria solani* isolates from Gudmestad et al. 2013.

Disease control of D123E-mutant *A. solani* isolates under the application of boscalid and fluopyram in vivo. Independent analysis of in vivo disease control experiments for boscalid and fluopyram determined that variances were homogenous (P=0.05) and experiments were combined for further analysis. In the analysis of experimental data evaluating each fungicide, a significant interaction was observed between the main and subplot factors (isolate and fungicide concentration, respectively) ($P < 0.0001$) for percentage disease control. Significant effects ($P < 0.0001$) were also observed for isolate and fungicide concentration for experiments evaluating percentage disease control provided by boscalid and fluopyram.

After the application of boscalid, significantly lower levels of disease control were observed for D123E-mutant isolates compared to SDHI sensitive isolates at concentrations of 0.1, 1, 10, and 100 µg/ml (Table 2.4). Area under the dose response curve (AUDRC) values for all five D123E-mutant isolates, ranging from 8840.2 to 8298.7, were also significantly lower compared to SDHI-sensitive isolates, ranging from 9757.5 to 9718.0, indicating reduced disease control in vivo for *A. solani* isolates possessing the D123E mutation. Five *A. solani* isolates possessing the H278R, H134R, or H133R mutations conferring SDHI resistance, but a moderate level of resistance to boscalid in vitro (1174-9, 1231-9, 1172-6, 1172-8, and 1176-3) (Table 2.4), were also found to have significantly reduced disease control in vivo at boscalid concentrations of 0.1, 1, 10 , and 100 µg/ml compared to SDHI-sensitive isolates. Dose response curves for boscalid indicate that D123E-mutant *A. solani* isolates and isolates possessing the H278R, H134R, or H133R mutations were controlled similarly at all fungicide concentrations (Table 2.4; Fig. 2.2A).

Table 2.4. Mean in vivo percentage disease control of *Alternaria solani* isolates by boscalid as determined in greenhouse assays.

			Boscalid concentration (µg/ml)				
Isolate	Mutation present[w]	Boscalid EC_{50}[x](µg/ml)	0.1	1	10	100	AUDRC[y]
1342-8	SDHI-sens.	4.01	40.0 a	76.1 b	97.6 a	100.0 a	9727.7 a
1331-6	SDHI-sens.	3.12	40.0 a	77.1 ab	98.0 a	100.0 a	9749.7 a
1393-5	SDHI-sens.	4.12	39.9 a	77.4 a	98.1 a	100.0 a	9757.5 a
1393-8	SDHI-sens.	3.21	39.1 b	77.7 a	97.3 a	100.0 a	9718.0 a
1486-11	SDHI-sens.	3.01	39.2 b	78.0 a	97.9 a	100.0 a	9750.0 a
1172-8	H133R	6.28	18.8 f	44.4 f	76.6 fg	95.4 e	8313.6 f
1176-3	H133R	6.80	18.9 f	45.1 f	77.4 ef	97.7 d	8456.3 e
1174-9	H134R	5.89	19.8 de	53.0 c	83.3 b	100.0 a	8894.7 b
1231-9	H134R	3.34	20.4 d	53.5 c	82.9 b	100.0 a	8877.4 b
1172-6	H278R	5.01	22.9 c	53.1 c	81.1 c	98.5 c	8717.1 c
1342-6	D123E	4.81	18.8 f	44.8 f	77.9 e	97.5 d	8470.5 e
1332-2	D123E	7.34	19.6 e	51.3 d	83.2 b	99.1 b	8840.2 b
1364-1	D123E	4.54	18.8 f	46.7 e	77.8 e	97.4 d	8470.7 e
1393-1	D123E	4.21	22.5 c	53.4 c	79.6 d	97.3 d	8590.6 d
1393-18	D123E	6.18	18.6 f	44.2 f	76.3 g	95.4 e	8298.7 f
$LSD_{P=0.05}$[z]			0.7	1.1	1.0	0.5	56.8

[w] Mutation presence conferring SDHI (D123E, H133R, H134R, and H278R) and QoI (F129L) resistance.

[x] EC_{50} (the effective concentration at which the fungal growth is inhibited by 50%) values to boscalid from in vitro assays.

[y] AUDRC = Area under dose response curve.

[z] Least significant difference at the R = 0.05 level.

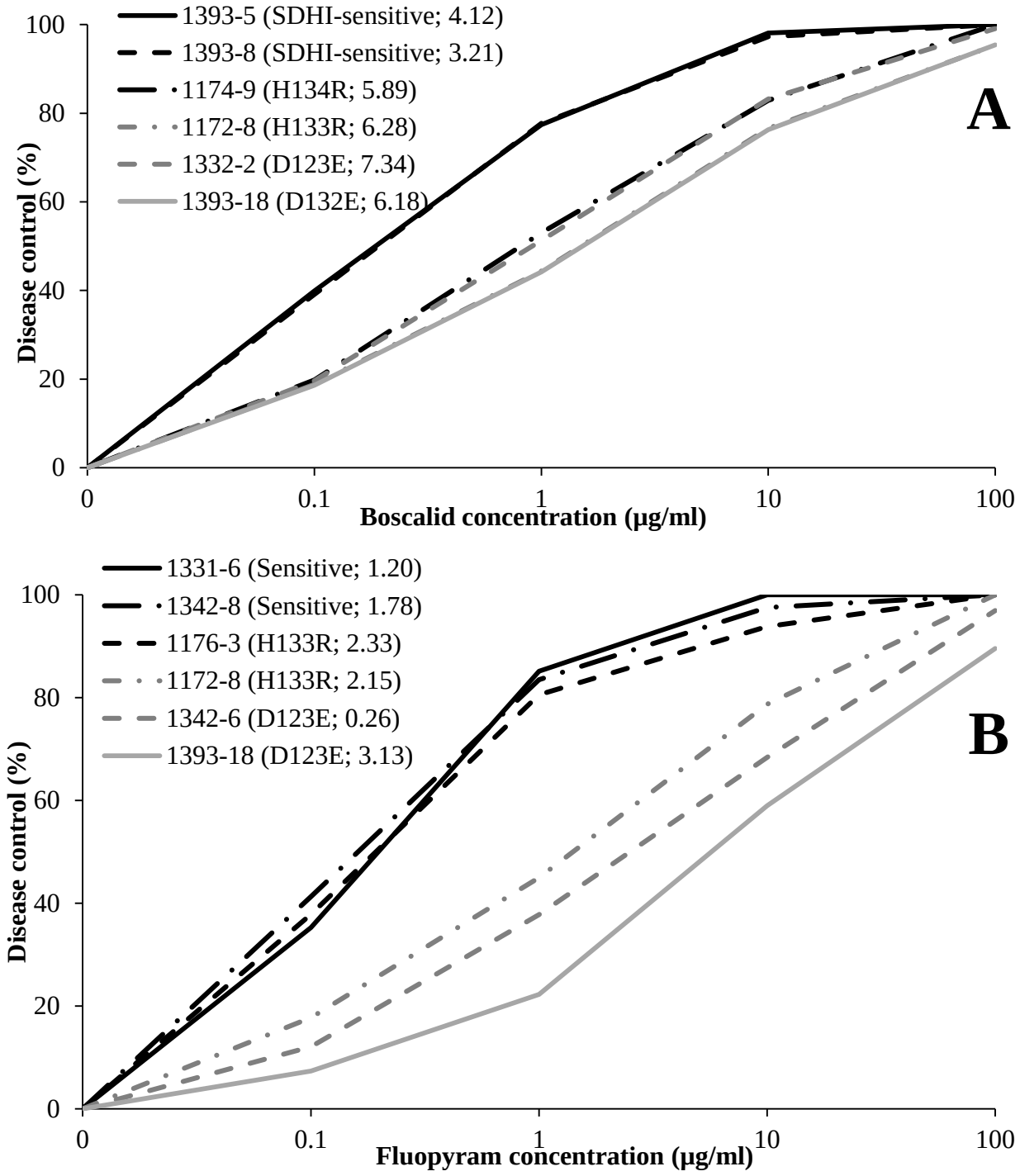

Fig. 2.2 Mean in vivo percentage disease control of *Alternaria solani* isolates by **A**, Boscalid and **B**, Fluopyram as determined in greenhouse assays. The six out of fifteen isolates in the dose response curve represent the highest and lowest sensitivities to each fungicide of SDHI-sensitive isolates, SDHI-mutant isolates possessing the H133R, or H134R, and D123E-mutant isolates, respectively.

Significant differences in disease control under the application of fluopyram were also observed among *A. solani* isolates in vivo. Percentage disease control at each concentration of fluopyram was significantly lower for D123E-mutant isolates compared to SDHI-sensitive isolates, as well as isolates possessing mutations conferring SDHI resistance such as the H278R, H134R, and H133R (Table 2.5). All five D123E-mutant isolates evaluated were determined to have significantly reduced disease control compared to all other *A. solani* isolates evaluated across concentrations of 0.1, 1, and 10 µg/ml. AUDRC values for D123E-mutant isolates, ranging from 7938.8 to 7064.3, were significantly lower compared to SDHI-sensitive isolates, ranging from 9887.4 to 9760.5, and isolates possessing the H278R, H134R, or H133R mutations, ranging from 9761.4 to 8627.2, indicating significantly reduced disease control of D123E-mutant isolates by fluopyram in vivo (Table 2.5). Based on these data, disease control provided by fluopyram of every *A. solani* isolate with the D123E mutation was significantly lower than disease control of SDHI-sensitive isolates and isolates possessing the H134R, H278R, and H133R mutations (Table 2.5; Fig. 2.2B). Even at the highest concentration of 100 µg/ml of fluopyram, disease control of three out of five D123E-mutant *A. solani* isolates (1332-2, 1364-1, and 1393-18) evaluated in vivo was significantly lower than the disease control of SDHI-sensitive isolates and isolates possessing the H134R, H278R, or H133R mutations (Table 2.5).

Table 2.5. Mean in vivo percentage disease control of *Alternaria solani* isolates by fluopyram as determined in greenhouse assays.

			Fluopyram concentration (µg/ml)				
Isolate	Mutations present[w]	Fluopyram EC_{50}[x](µg/ml)	0.1	1	10	100	AUDRC[y]
1342-8	SDHI-sens.	1.78	41.3 a	83.5 ab	97.6 ab	100.0 a	9760.5 b
1331-6	SDHI-sens.	1.20	35.3 c	85.2 ab	100.0 a	100.0 a	9887.4 a
1393-5	SDHI-sens.	2.08	36.9 bc	83.9 ab	100.0 a	100.0 a	9881.8 a
1393-8	SDHI-sens.	0.39	37.8 bc	82.5 ab	100.0 a	100.0 a	9875.4 a
1486-11	SDHI-sens.	0.45	39.7 ab	83.3 ab	99.0 ab	100.0 a	9830.7 ab
1172-8	H133R	2.15	17.7 d	45.0 c	78.7 e	100.0 a	8627.2 e
1176-3	H133R	2.33	37.8 bc	80.6 b	93.8 c	100.0 a	9559.6 c
1174-9	H134R	2.09	17.6 d	46.1 c	82.0 d	97.9 ab	8700.3 e
1231-9	H134R	1.55	19.0 d	49.2 c	83.4 d	100.0 a	8881.1 d
1172-6	H278R	0.11	35.0 c	86.3 a	97.4 b	100.0 a	9761.4 b
1342-6	D123E	0.26	12.1 e	37.8 d	68.4 f	96.9 ab	7938.8 f
1332-2	D123E	1.74	11.4 e	35.6 d	67.5 f	96.5 b	7866.1 f
1364-1	D123E	0.37	11.3 e	36.8 d	67.5 f	95.8 b	7837.1 f
1393-1	D123E	2.46	9.4 e	36.4 d	67.6 f	97.1 ab	7901.3 f
1393-18	D123E	3.13	7.3 f	22.3 e	59.0 g	89.6 c	7064.3 g
$LSD_{P=0.05}$[z]			3.3	4.9	2.5	3.2	112.1

[w] Mutation presence conferring SDHI (D123E, H133R, H134R, and H278R) and QoI (F129L) resistance.

[x] EC_{50} (the effective concentration at which the fungal growth is inhibited by 50%) values to fluopyram from in vitro.

[y] AUDRC = Area under dose response curve.

[z] Least significant difference at the R = 0.05 level.

Effect of the D123E mutation on early blight disease control under field conditions.

Results from field trials conducted in northeast North Dakota in 2013 and 2015 demonstrated that there were no differences among fungicide treatments in the control of early blight (Fig. 2.3). However, in both years of the trial, all fungicide treatments provided significant control of early blight compared to the non-treated control plots (Treatment 1).

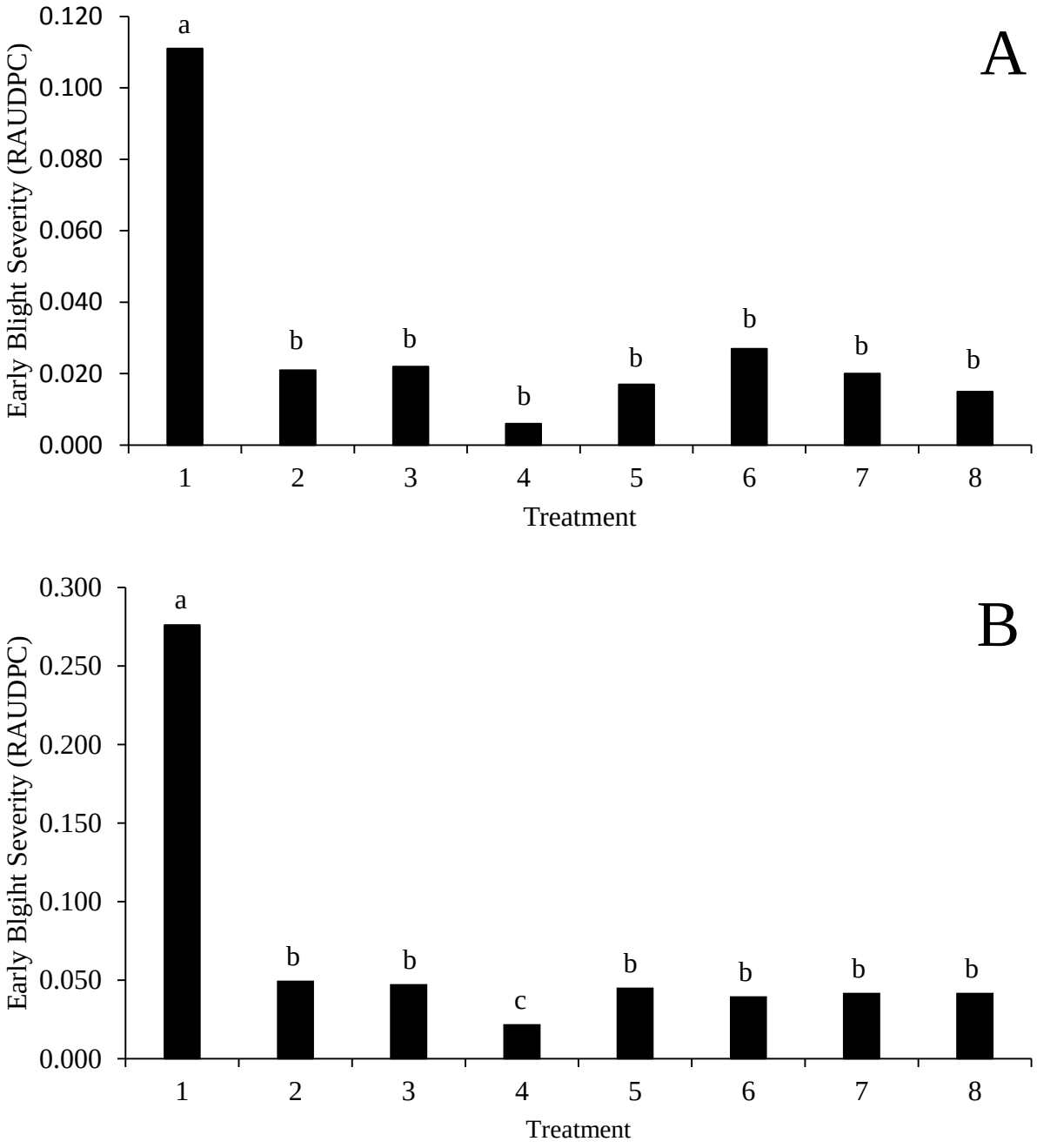

Fig. 2.3. Early blight severity, expressed as relative area under the disease progress curve (RAUDPC) from a field trial conducted in (**A**) 2013 and (**B**) 2015 in Inkster, ND. Treatments included a non-treated control (1); Chlorothalonil (2); Mancozeb (3); Separate applications of QoI, SDHI, and PA fungicides each mixed with standard protectants (4); fluopyram in-furrow (5); fluopyram in-furrow with a low and high rate of *Bacillus subtilis* (6 and 7, respectively); and imidacloprid in-furrow mixed with fluopyram and *Bacillus subtilis* (8).

The prevalence of *A. solani* isolates that possess the D123E mutaton was significantly higher ($P < 0.0001$) in treatments receiving an application of fluopyram in furrow (Fig. 2.4). In fact, in both years, none of the isolates collected from non-treated plots or plots receiving foliar applications of chlorothalonil and mancozeb possessed the D123E mutation (Fig. 2.4). In contrast, in experimental units receiving fluopyram in-furrow, the frequency of D123E-mutant isolates collected ranged from 0 to 35% in 2013, and from 5 to 37.5% in 2015. When fluopyram was applied alone and in furrow at planting (Treatment 5), the highest frequency of D123E-mutant isolates was 35% in 2013 and 37.5% in 2015. Frequency of D123E-mutants in treatments receiving *Bacillus subtilis* (Serenade Soil™) in-furrow in combination with fluopyram (Treatments 6 and 7) was also significantly higher than all other treatments. Interestingly, when Serenade Soil was applied in combination with fluopyram in-furrow at planting, the frequency of the D123E mutation in *A. solani* isolates recovered was significantly lower ($P < 0.0001$) compared to fluopyram applied in-furrow alone (Fig. 2.4). The rate of *Bacillus subtilis* applied with fluopyram also appeared to have a significant effect on the frequency of the D123E mutation detected at the end of the season. When a low rate of Serenade Soil (4.7 liters/hectare) was applied in combination with fluopyram in-furrow (Treatment 6), the frequency of *A. solani* isolates with the D123E mutation was 20% and 15% in 2013 and 2015, respectively, compared to 0% and 5% of isolates detected when a higher rate of Serenade Soil (9.4 liters/hectare) (Treatment 7) was used in combination with fluopyram in furrow at planting (Fig. 2.4).

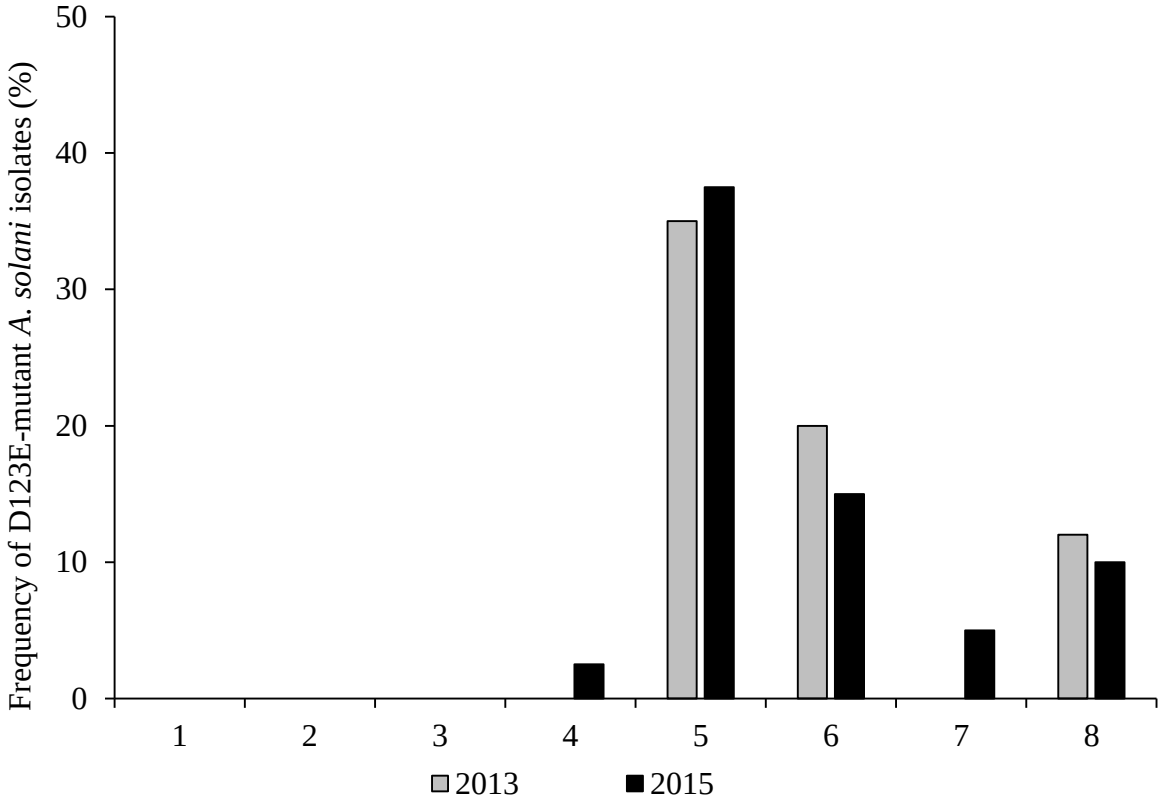

Fig. 2.4. The frequency of D123E-mutant *Alternaria solani* isolates recovered from each treatment in the field in 2013 and 2015. Treatments included a non-treated control (1); Chlorothalonil (2); Mancozeb (3); Separate applications of QoI, SDHI, and AP fungicides each mixed with standard protectants (4); Fluopyram in-furrow (5); Fluopyram in-furrow with a low and high rate of *Bacillus subtilis* (6 and 7, respectively); and imidacloprid in-furrow mixed with fluopyram and *Bacillus subtilis* (8).

Discussion

This study is the first to characterize reduced sensitivity to fluopyram in *A. solani* using both in vivo and field experiments. The D123E mutation was previously characterized as one of five known point mutations on three *AsSdh* genes in *A. solani* known to independently confer resistance to the SDHI fungicides boscalid and penthiopyrad (Mallik et al. 2014). In the previous study, only a single isolate out of 67 characterized was identified as possessing the D123E mutation. This first identified D123E-mutant *A. solani* isolate was collected from Nebraska in 2011 and determined to have very high resistance to boscalid and penthiopyrad, but was

determined to be sensitive to fluopyram (Mallik et al. 2014). This was supported by a number of studies that have identified a lack of cross-resistance between SDHI fungicides boscalid and fluopyram in *A. solani,* and determined that isolates with high levels of boscalid resistance were sensitive to fluopyram (Fairchild et al. 2012; Gudmestad et al. 2013; Miles et al. 2014). In a subsequent survey conducted by our laboratory through the years of 2013 through 2015, *A. solani* isolates possessing the D123E mutation were identified at much higher frequencies and from multiple states including Colorado, Michigan, Minnesota, Nebraska, North Dakota, and Texas (Bauske et al. Plant Disease in review). D123E-mutant isolates also increased in frequency in several of these states every year of the survey and comprised 12% of total isolates collected in 2015 (Bauske et al. Plant Disease in review). This study was conducted to assist our understanding of the observed increase in the frequency of D123E mutations in *A. solani* isolates.

In 2013, when *A. solani* isolates were collected from multiple treatments in field trials and characterized in our laboratory for the presence of mutations conveying resistance to SDHI fungicides, it was determined that the frequency of *A. solani* isolates possessing the D123E mutation was significantly higher ($P < 0.0001$ according to χ^2 analysis) in treatments where fluopyram had been applied in-furrow. In 2015, when the trial was repeated, similar results were obtained. Despite the fact that *A. solani* isolates possessing the D123E mutation were not included in the inoculum mixture used to inoculate the trials in either year, their prevalence was high in plots receiving fluopyram in-furrow. As an explanation of this increased occurrence, it was initially thought that *A. solani* isolates possessing the D123E mutation would have higher EC_{50} values to fluopyram in vitro and therefore, reduced sensitivity to fluopyram. As previously disscussed, although most D123E-mutant isolates were found to confer high resistance to

boscalid, all were found to have EC_{50} values well below 5 µg/ml to fluopyram and determined to be sensitive to the fungicide on an in vitro basis. Although most *A. solani* isolates possessing the D123E mutation evaluated were found to be highly resistant to boscalid in vitro, 20% of isolates with the D123E mutation were found to be sensitive with EC_{50} values of around 5 µg/ml to boscalid. Resistance to boscalid and sensitivity to fluopyram in *A. solani* isolates with the D123E mutation in vitro may be due to fluopyram having a slightly different binding site compared to other SDHIs such as boscalid and penthiopyrad, rendering fluopyram activity unaffected by the same mutations (Avenot and Michailides, 2010; Gudmestad et al. 2013; Miles et al. 2014).

Five D123E-mutant *A. solani* isolates, five SDHI-sensitive isolates, and five *A. solani* isolates possessing other mutations conferring SDHI resistance were chosen for in vivo studies based on similar EC_{50} values in vitro to both boscalid and fluopyram. Although all fifteen *A. solani* isolates chosen for greenhouse assessment had similar EC_{50} values to boscalid and fluopyram in vitro, significant differences among isolates were observed in disease control provided by both fungicides in vivo. Percentage disease control provided by boscalid was not significantly different among *A. solani* isolates possessing the D123E mutation and isolates possessing the H133R mutation, but isolates possessing either of these mutations were controlled less with boscalid application compared to isolates possessing the H134R or H133R mutations, although not always significantly. This study suggested that *A. solani* isolates possessing either of these mutations may be associated with reduced disease control under boscalid, even though EC_{50} values in vitro suggests sensitivity to boscalid. Under the application of fluopyram, percentage disease control was significantly lower for all D123E-mutant isolates compared to *A. solani* isolates possessing other SDHI mutations such as the H133R, H134R, or the H278R, regardless of similar EC_{50} values to fluopyram in vitro. It is apparent from the study reported

here that under in vivo conditions isolates of *A. solani* with the D123E mutation are not controlled with either boscalid or fluopyram. Even *A. solani* isolates with extremely high sensitivity to fluopyram in vitro, such as isolates 1342-6 and 1364-1 (EC_{50} = 0.26 and 0.38 µg/ml, respectively), expressed significantly reduced disease control in vivo compared to isolates possessing the H134R, H133R, or H278R mutations and SDHI-sensitive isolates. This suggests that isolates with the D123E mutation have a selective advantage under selection pressure from fluopyram compared to SDHI-sensitive isolates and other SDHI-mutant isolates possessing the H278R, H134R, or H133R mutation, although it is unclear what that selective advantage might be.

The most interesting outcome of these studies was that *A. solani* isolates possessing the D123E mutation were recovered at significantly higher frequencies from treatments where fluopyram was applied in-furrow at planting in two separate years. Although disease severity among treatments receiving fluopyram in furrow and treatments receiving foliar applications of fungicides was not significantly different either year, the genetic makeup of the isolates recovered from those treatments was different. As previously discussed, none of the four isolates used in field inoculations possessed the D123E mutation, and none of the isolates collected from non-inoculated plots, or from plots receiving multiple applications of the standard protectants chlorothalonil and mancozeb, were found to possess the D123E mutation in either year. However, 35% and 37.5% of isolates collected from plots receiving fluopyram alone in-furrow possessed the D123E mutation in 2013 and 2015, respectively, suggesting the existence of a selective advantage of *A. solani* isolates possessing the D123E mutation with a selection pressure of fluopyram under field conditions. Since fluopyram was registered in the United States in early 2012, very few studies have identified any resistance. However, *B. cinerea* isolates possessing

the H272Y or H272L mutation in the *BcSdhB* gene recovered from strawberry were shown to have reduced sensitivity to the chemistry (Amiri et al. 2014). Low and moderate levels of resistance to fluopyram have also been detected in *A. alternata* isolates collected from peach orchards in South Carolina (Yang et al. 2015). Given the ability of *A. solani* to develop resistance to QoI, SDHI, and AP fungicides (Pasche et al. 2004; 2005; Gudmestad et al. 2013; Fairchild et al. 2013; Fonseka and Gudmestad, 2016), it is highly likely that resistance to fluopyram will further develop and be characterized beyond the selective advantage reported here. The application of fluopyram in-furrow, while initially providing excellent season-long early blight control, places constant selection upon the *A. solani* population due to the systemic activity of the fungicide. Fluopyram has been previously determined to be highly xylem systemic when applied in-furrow and translocated to the growing point of the plant (Laleve et al. 2013), possibly due to high water solubility that is favorable for translocation (Labourdette et al. 2011).

One of the most important factors to consider in the development of fungicide resistance is the parasitic fitness of resistant isolates in the presence and absence of selection pressure (Dekker, 1981). Fitness can be defined as the survival or reproductive success of an allele, individual, or group (Pringle and Taylor, 2002), and if a resistant subpopulation of a pathogen is more prevalent, fitness costs may be absent and differences in parasitic fitness between fungicide-resistant and -sensitive isolates may exist (Fan et al. 2015). One reason for the observed selection advantage of *A. solani* isolates possessing the D123E mutation under fluopyram in this study may be increased parasitic fitness of D123E-mutant isolates under selection pressure. A selective disadvantage or reduced fitness of *A. alternata* isolates that possess the D123E mutation on the *AaSdhD* gene has previously been speculated because isolates with this mutation were recovered at very low frequencies (Avenot et al. 2009; Avenot

and Michailides, 2010). While initially recovered at very low frequencies in previous studies (Mallik et al. 2014), the increased frequency of *A. solani* isolates possessing the D123E mutation may suggest the absence of fitness penalties in these isolates. While *A. solani* isolates possessing the F129L mutation conferring resistance to QoI fungicides were previously reported to have reduced spore germination in vitro compared to wild-type isolates, the same study also determined that isolates with the F129L mutation had increased aggressiveness in vivo, indicating the absence of any substantial fitness penalties (Pasche and Gudmestad, 2008). Similarly, no evidence of major fitness penalties due to mutations in the *SdhB* gene in SDHI-resistant *B. cinerea* isolates recovered from strawberry were identified (Amiri et al. 2014). Other research has reported increased sensitivity to oxidative stress in *A. alternata* isolates possessing mutations conferring SDHI resistance, including isolates possessing the D123E mutation (Fan et al. 2015; Avenot et al. 2009), but no significant fitness penalties, suggesting that resistant isolates would likely compete successfully under field conditions. However, nothing is known about the fitness of *A. solani* isolates possessing the D123E mutation in the absence of selection pressure and future research will focus on the characterization of any fitness penalties associated with mutations conferring SDHI resistance.

This report clearly demonstrated that *A. solani* isolates possessing the D123E mutation have a selective advantage under the application of fluopyram compared to wild-type *A. solani* isolates and isolates possessing other mutations conferring SDHI resistance, such as the H278R, H134R, and H133R. In the studies reported here, we demonstrate the lack of disease control of *A. solani* isolates with the D123E mutation under the application of fluopyram in vivo and identified a significantly higher prevalence of isolates possessing the D123E mutation in treatments receiving fluopyram in-furrow at planting in the field. These data suggest that

applying fluopyram in-furrow for early blight control in commercial potato fields would lead to the further increase of D123E-mutant *A. solani* isolates and in turn lead to reduced disease control. Future research will need to evaluate the fitness of *A. solani* isolates possessing mutations conferring SDHI resistance in the absence of fungicide selection pressure. Furthermore, since reduced sensitivity of *A. solani* to pyrimethanil was detected in field isolates collected in 2010 from Idaho (Fairchild et al. 2013) and further studies have shown reduced sensitivity to pyrimethanil in vivo (Fonseka and Gudmestad, 2016), monitoring for and assessing the fitness of *A. solani* isolates resistant to three chemical classes will be crucial.

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CHAPTER 3. PARASITIC FITNESS OF FUNGICIDE-RESISTANT AND -SENSITIVE ISOLATES OF *ALTERNARIA SOLANI*

Abstract

Succinate dehydrogenase inhibiting (SDHI) and quinone outside inhibiting (QoI) fungicides are widely applied in commercial potato fields for the management of early blight, caused by *Alternaria solani*. Resistance to SDHIs in *A. solani* has been attributed to five known point mutations on three *AsSdh* genes, while resistance to QoIs is conferred by the substitution of phenylalanine with leucine at position 129 (F129L) in the *cytb* gene. The objective of this study was to investigate the parasitic fitness of *A. solani* isolates possessing mutations associated with resistance to one or both of these chemical classes. A total of 120 *A. solani* isolates collected from various geographical locations around the United States were chosen for in vitro assessment, and 60 of these isolates were further evaluated in vivo. An additional six *A. solani* isolates previously determined to have reduced sensitivity to the pyrimethanil (anilino-pyrimidine) but also possessing QoI and SDHI resistance, were included in in vitro and in vivo fitness experiments. Fitness parameters measured were (i) spore germination in vitro, (ii) mycelial expansion in vitro, and (iii) aggressiveness in vivo. No significant differences in spore germination or mycelial expansion ($P = 0.44, 0.69$, respectively) were observed among grouped SDHI-resistant and SDHI-sensitive isolates in vitro. Only *A. solani* isolates possessing the D123E mutation were shown to be significantly more aggressive in vivo ($P = <0.0001$) compared to SDHI-sensitive isolates. These results indicate that SDHI-resistant *A. solani* isolates have no significant fitness penalties compared to sensitive isolates under the parameters evaluated regardless of the presence or absence of reduced sensitivity to QoI or anilino-pyrimidine reduced sensitivity. Results of these studies suggest that *A. solani* isolates with

multiple fungicide resistances may compete successfully with fungicide-sensitive isolates under field conditions.

Introduction

Early blight, caused by the fungal pathogen *Alternaria solani* Sorauer, is an economically important foliar disease of potato (*Solanum tuberosum* L.) in many production areas across the United States. Since few commercially acceptable potato cultivars possess resistance, the most effective early blight management tactic is the frequent application of foliar fungicides from early in the growing season until vine desiccation (Gudmestad et al. 2013; Pscheidt and Stevenson, 1988). Locally systemic and translaminar fungicides such as quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), and anilino-pyrimidines (APs) are widely applied for the management of early blight and achieve control at higher levels of disease pressure compared to standard protectant fungicides such as chlorothalonil and mancozeb (Pasche and Gudmestad; 2008; Yellareddygari et al. 2016).

The QoI fungicides, which include chemistries such as azoxystrobin, trifloxystrobin, and pyraclostrobin, have a single site mode of action, interfering with the electron transport of the cytochrome bc_1 complex (Bartlett et al. 2002), thereby inhibiting fungal respiration at mitochondrial complex III. Following their introduction in 1999, the QoIs initially provided high levels of disease control, but reduced efficacy was reported within two years due to increased selection pressure placed on the pathogen by extensive usage (Pasche et al. 2004). Resistance to QoI fungicides in many pathogens is attributed to the substitution of glycine with alanine at position 143 in the cytochrome b (*cytb*) gene (Bolton et al. 2013; Ishii et al. 2001; Koller et al. 2001; Sierotzki et al. 2000), but reduced sensitivity in *A. solani* has been shown to result from the substitution of phenylalanine with leucine at position 129 in the *cytb* gene (Pasche et al.

2005). By 2006, *A. solani* isolates possessing the F129L mutation were prevalent throughout much of the United States (Pasche and Gudmestad, 2008).

The primary target site of SDHI fungicides is mitochondrial complex II at either the succinate ubiquinone reductase or succinate dehydrogenase complex, where molecules of this class of fungicide bind and inhibit fungal respiration (Avenot and Michailides, 2010; Sierotzki and Scalliet, 2013). The widespread application of SDHI fungicides, beginning in 2005 with the registration of boscalid, placed significant selection pressure on pathogen populations, and mutations associated with SDHI resistance have been identified in *Alternaria alternata*, *Botrytis cinerea*, *Corynespora cassiicola*, *Didymella bryoniae*, *Podosphaera xanthii*, and *A. solani* (Avenot et al. 2008, 2010, 2012; Bardas et al. 2010; Ishii et al. 2011; Mallik et al. 2014) among others. Five known point mutations, including substitutions of histidine to tyrosine (H278Y) or arginine (H278R) at codon 278 in the *AsSdhB* gene, a substitution of histidine to arginine (H134R) at codon 134 in the *AsSdhC* gene, and substitutions of histidine to arginine (H133R) at codon 133 and aspartic acid to glutamic acid (D123E) at codon 123 in the *AsSdhD* gene, have been identified to independently confer moderate to high levels of resistance to SDHI fungicides in *A. solani* (Mallik et al. 2014). A recent study in our laboratory determined that, in a diverse population of *A. solani* isolates collected across several years from 11 states, over 95% of isolates possessed mutations conferring SDHI resistance, and nearly all of those SDHI-resistant isolates also possessed the F129L mutation conferring resistance to QoI fungicides (Bauske et al. Plant Disease in review).

The single-site mode of action of AP fungicides has been suggested to result in the inhibition of the secretion of fungal hydrolytic enzymes required during infection and inhibit the biosynthesis of methionine (Heye et al. 1994; Masner et al. 1994). APs have been used for the

control of several pathogens including *B. cinerea* (Zhao et al. 2010) and *Venturia inaequalis* (Koller et al. 2005). However, resistance to APs has been reported in field isolates of *B. cinerea* of various crops (Amiri et al. 2013; Leroux et al. 1999; Myresiotis et al. 2007), *Pecillium* spp. of apple and citrus (Kanetis et al. 2008; Xiao et al. 2011), as well as *V. inaequalis* of apple (FRAC, 2015). In 2005, the AP pyrimethanil was registered for early blight control in potato. Unfortunately, in 2010, resistance was reported in *A. solani* isolates recovered in Idaho, with 19% of isolates collected determined to be reduced sensitive to the fungicide (Fairchild et al. 2013). A recent study determined that 6 out of 245 *A. solani* isolates collected from 2010 to 2014 possessed reduced sensitivity to pyrimethanil in vitro and additionally found that reduced sensitive isolates were not controlled at most pyrimethanil doses in greenhouse efficacy assays (Fonseka and Gudmestad, 2016).

The development of fungicide resistance in the field depends upon a number of different factors including presence of selection pressure, presence of a susceptible host, fungicide dosage, application intervals, persistence of fungicide applied, and fungicide application method (Peever and Milgroom, 1995). However, one of the most important factors to consider in the development of fungicide resistance is the parasitic fitness of resistant isolates in the presence and absence of selection pressure (Dekker, 1981). Parasitic fitness can be defined as the relative ability of a parasitic genotype or population to persist over time and contribute to the future gene pool (MacHardy et al. 2001) and is quantifiable using a number of different parameters including infection efficiency and amount of disease caused, or aggressiveness. Fitness costs can also be measured in terms of both predicted fitness (mycelial growth, spore germination, and spore production in vitro) and realized fitness (in vivo and competitive experiments) (Antonovics and Alexander, 1989). Mutations associated with fungicide resistance may display deleterious

pleiotrophic effects, or fitness penalties, that become apparent in the absence of fungicide selection pressure (Jeger et al. 2008; Karaoglanidis and Michailides, 2011). Many of the studies aimed at characterizing fitness penalties associated with fungicide resistance development have conflicting results, thus making definitive conclusions on the persistence of fungicide resistance difficult (Karaoglanidis and Michailides, 2011). A limited amount of studies have identified substantial fitness penalties in resistant isolates in both laboratory and field experiments (Iacomi-Vasilescu et al. 2008; Kadish and Cohen, 1992; Karaoglanidis et al. 2001). Most research has indicated that parasitic fitness penalties are insignificant (Billard et al. 2012; Kim and Xiao, 2011) or completely absent (Corio-Costet et al. 2010; Peever and Milgroom, 1994) in fungicide-resistant populations.

Due to the findings that a significant proportion of the *A. solani* population contains dual resistance to SDHI and QoI fungicides (Gudmestad et al. 2013; Bauske et al. Plant Disease in review), and pyrimethanil resistance has been documented in a diverse *A. solani* population (Fonseka and Gudmestad, 2016), detailed knowledge of the fitness of resistant isolates is crucial to optimize fungicide resistance management strategies and achieve effective early blight disease management. The objectives of the research reported here were to (i) determine the predicted fitness of *A. solani* isolates possessing resistance to one or more chemical classes in vitro; (ii) and determine the realized fitness of *A. solani* isolates possessing resistance to one or more chemical classes in vivo.

Materials and Methods

***A. solani* isolate selection.** A total of 120 *A. solani* isolates were evaluated during these studies. Isolates were selected based on origin and the presence or absence of mutations conferring fungicide resistance. *A. solani* isolates recovered from eight states including North

Dakota, Minnesota, Nebraska, Texas, Colorado, Michigan, Idaho, and Wisconsin from 2011 through 2015 were selected (Table 3.1; Table 3.2). Twenty *A. solani* isolates selected possess none of the five mutations conferring SDHI resistance and were grouped together as SDHI-sensitive isolates. The additional 100 of the 120 total isolates were grouped based on the presence of each mutation (H278Y, H278R, H134R, H133R, and D123E). There were 20 isolates in each of the five mutation groups (Table 3.1). The 120 individual isolates evaluated in vitro were first grouped for analysis on the basis of presence and absence of mutations conferring SDHI resistance only (Table 3.1). Isolates selected were further grouped for additional analysis based on resistance to one, two, or three chemical classes (Table 3.2). Also included were six isolates previously determined to be reduced sensitive to the anilino-pyrimidine fungicide pyrimethanil (Fonseka and Gudmestad, 2016) and possessing mutations conferring SDHI and QoI resistance, thus resistant to three chemical classes.

Table 3.1. Origin of *Alternaria solani* isolates used in in vitro fitness experiments.

Mutation	State of origin	No. of isolates
Sensitive[z]	North Dakota	10
	Minnesota	7
	Nebraska	1
	Colorado	1
	Texas	1
H278Y	North Dakota	6
	Minnesota	3
	Nebraska	4
	Texas	5
	Colorado	1
	Idaho	1
H278R	North Dakota	8
	Minnesota	1
	Nebraska	3
	Texas	1
	Colorado	3
	Idaho	2
	Wisconsin	2
H134R	North Dakota	6
	Minnesota	2
	Nebraska	4
	Texas	5
	Colorado	2
	Idaho	1
H133R	North Dakota	2
	Minnesota	3
	Nebraska	5
	Texas	1
	Colorado	2
	Michigan	3
	Idaho	4
D123E	North Dakota	6
	Minnesota	1
	Nebraska	7
	Texas	3
	Colorado	1
	Michigan	2
Total	-	120

[z] *Alternaria solani* isolates that possess no known mutations associated with SDHI resistance.

Table 3.2. Number of *Alternaria solani* isolates in each resistance group.

Mutation[z]	State of origin	No. of isolates
Wild Type	North Dakota	3
	Colorado	1
F129L	North Dakota	7
	Minnesota	7
	Nebraska	1
	Texas	1
H278Y	North Dakota	3
	Nebraska	2
	Idaho	1
H278R	North Dakota	4
	Nebraska	1
	Idaho	1
H134R	North Dakota	2
	Nebraska	1
	Texas	1
H133R	North Dakota	1
	Colorado	1
D123E	North Dakota	2
	Nebraska	3
H278Y/F129L	North Dakota	3
	Minnesota	2
	Nebraska	2
	Texas	4
H278R/F129L	North Dakota	4
	Minnesota	1
	Colorado	3
	Nebraska	2
	Texas	1
	Idaho	1
	Wisconsin	2
H134R/F129L	North Dakota	4
	Minnesota	2
	Colorado	2
	Nebraska	3
	Texas	3
	Idaho	1
H133R/F129L	North Dakota	1
	Minnesota	3
	Nebraska	5
	Colorado	1

Table 3.2. Number of *Alternaria solani* isolates in each resistance group (continued).

Mutation[z]	State of origin	No. of isolates
	Texas	1
	Michigan	3
	Idaho	2
D123E/F129L	North Dakota	4
	Minnesota	1
	Nebraska	4
	Texas	3
	Colorado	1
	Michigan	2
H278Y/F129L/P	Colorado	1
	Minnesota	1
	Texas	1
H134R/F129L/P	Texas	1
H133R/F129L/P	Idaho	2
Total	-	120

[z] H278Y, H278R, H134R, H133R, and D123E mutation confer resistance to SDHI fungicides, the F129L mutation confers resistance to QoI fungicides, and AP denotes reduced sensitivity to the anilino-pyrimidine fungicide pyrimethanil.

Predicted fitness of fungicide-resistant and -sensitive *A. solani* isolates. *Spore germination*. Conidial germination assays were conducted as previously described (Pasche et al. 2005; Gudmestad et al. 2013) using 7- to 14-day-old cultures of *A. solani* maintained under continuous fluorescent light at 22 ± 2°C. A glass rod was used to free conidia from the surface of the CV8 media using sterile, distilled water under aseptic conditions. The conidial concentration of the suspension was determined using a hemocytometer and adjusted to 2×10^4 conidia/ml by adding sterile, distilled water. A 150 µl aliquot of the conidial suspension then was added to media containing 2% laboratory grade agar (A360-500 Fisher Scientific, Pittsburgh, PA). Inoculated plates were incubated at 21°C under continuous light for 4 h prior to evaluation of conidial germination (Pasche et al. 2004). Following incubation, 50 conidia/plate were examined for the development of one germ tube at least as long as the conidium, or multiple germ tubes developing from one conidium, using a microscope at ×100 magnification. All experiments were

performed twice with three replicates for each isolate (Pasche et al. 2004; Karaoglanidis et al. 2011). The 120 *A. solani* isolates were tested for conidial germination in 12 trials, with 15 to 20 isolates included in each trial. Control isolates 13-1, a wild type *A. solani* isolate, and 526-3, a QoI-resistant isolate, were included in each trial as internal controls (Gudmestad et al. 2013).

Mycelial expansion. The identical fungicide-sensitive and -resistant isolates (Table 3.1) were also assessed for mycelial expansion in vitro. Agar sections from working cultures were transferred onto CV8 medium and were incubated under 24h fluorescent light at 22ºC (±2ºC). After 4 days, 5-mm agar plugs were excised from the leading edge of growth and were inverted onto 90mm petri plates containing 2% laboratory-grade agar. Two perpendicular measurements of mycelial growth for each isolate were measured, with the original plug diameter (5 mm) subtracted, after seven days incubation in the dark at 25ºC (±2ºC). Each *A. solani* isolate was evaluated a minimum of twice to determine mycelial expansion, with three replicates each trial (Fonseka and Gudmestad, 2016; Karaoglanidis and Michailides, 2011). The *A. solani* isolates 13-1 and 526-3, described above, were also included in each mycelial growth trial as internal controls.

Realized fitness of fungicide-resistant and -sensitive *A. solani* isolates.

Aggressiveness. The aggressiveness of 60 fungicide-sensitive and -resistant *A. solani* isolates previously included in in vitro experiments was evaluated under greenhouse conditions as previously described (Pasche and Gudmestad, 2008; Fonseka and Gudmestad, 2016). *A. solani* isolates were selected based on origin, mutation presence, and resistance to one or multiple fungicide classes. Isolates inoculated in greenhouse experiments were maintained for 7 to 14 days on CV8 media at 22°C ± 2°C under constant fluorescent light. A glass rod was used to free conidia from the surface of the CV8 media using sterile, distilled water. The conidial

concentration of the suspension was determined using a hemocytometer and adjusted to 2×10^5 conidia/ml. Tomato plants, cv. Orange Pixie VFT Hybrid (Tomato Growers Supply Company, Fort Myers, FL.), were grown in the greenhouse under natural light and ambient temperatures in 10 cm^3 pots containing Sunshine Mix LC1 (73 to 83% Canadian sphagnum peat moss, perlite, and dolomite lime). Three tomato seeds were sown in each pot and, following emergence, plants were thinned to obtain two uniformly sized plants/pot. Inoculations were performed when the first three leaves were fully expanded and plants were approximately 20 cm in height. Tomato plants were inoculated using 50 ml of conidial suspension/plant using a Preval paint-spray gun (Preval Sprayer Division; Precision Valve Corporation, Yonkers, NY) and inoculated plants were kept for 24 h at 22 ± 2°C in humid chambers (Phytotronic Inc., Earth City, MO). High relative humidity was achieved through in these chambers through mist application for 10 s every two min. Plants then were transferred to specially built confinement chambers with four walls and open ceilings on greenhouse benches at 25 ± 2°C to minimize inter-isolate interference and maintained with a daily application of water. The percentage disease severity, or percentage infected leaf area, on three fully-expanded true leaves on each of two plants/pot, with one pot/replicate and three replicates, was assessed visually at 6, 9, and 12 days post-inoculation (DPI) (Gudmestad et al. 2013). Each of the 60 isolates was evaluated in two separate trials and a total of 14 trials were conducted, with 7 to 10 isolates included in each trial. A control isolate 1342-8, a wild-type *A. solani* isolate, was included as an internal control in each trial as a measure of reproducibility.

Statistical analyses. All in vitro experiments were performed twice in completely random design with three replicates for each *A. solani* isolate. Levene's test was conducted to test for homogeneity of variance among two independent experiments (Milliken and Johnson,

1992). The isolates selected for this study were all field isolates and were divided into groups for ANOVA based on the presence of mutations. All 120 isolates assayed in vitro and the subset of 60 assayed in vivo were then analyzed divided into further resistance groups based on the F129L mutation presence and reduced sensitivity to pyrimethanil, in addition to mutations conferring SDHI resistance (Table 3.2). Percentage disease severity ratings were conducted in greenhouse assays as discussed above and foliar disease severity was used to calculate area under the disease progress curve (AUDPC) as follows (Shaner and Finney, 1977):

$$\mathrm{AUDPC} = \sum_{i=1}^{n} [(\mathrm{W}_{i+1} + \mathrm{W}_i)/2][\mathrm{t}_{i+1} - t_i],$$

Where W_i is the percentage foliar disease severity at the ith observation, t_i the time in days at the ith observation and n the total number of observations. The relative area under the disease progress curve (RAUDPC) was calculated for each treatment of the replicated trials from each year by dividing AUDPC values by the total area of the graph and analyzed using ANOVA (Proc GLM SAS version 9.3, Cary, NC). Fisher's protected LSD test (P = 0.05) was used to differentiate mean RAUDPC values (Pasche and Gudmestad, 2008). For in vitro experiments, analysis of variance (ANOVA) was performed on the combined results using PROC GLM in the Statistical Analysis System and differences among groups were compared using Fisher's protected least significant difference (LSD) test (P = 0.05). In all in vitro and in vivo studies control isolates were used in each trial as described above to determine reproducibility of assay. Assay reproducibility calculations were applied to the internal controls (Wong and Wilcox, 2002) generating limits for 95% confidence intervals for each internal control. Trials in which the data of the internal control for each assay were within the 95% confidence interval were included in further statistical analyses (Fonseka and Gudmestad, 2016; Wong and Wilcox, 2002).

Results

Determination of predicted fitness of fungicide-resistant and -sensitive *A. solani* isolates in vitro. *Spore germintation*. Mean spore germination percentage among groups ranged between 99.5% for D123E-mutants and 99.7% for H134R-mutants, but these groups were not significantly different, indicating similar spore germination among grouped SDHI-sensitive and -resistant isolates of *A. solani* (Table 3.3). There was also no significant difference in percentage spore germination when the isolates were categorized into additional groups based on resistance to multiple chemical classes (P = 0.99) (Table 3.2; Fig. 3.1). *A. solani* isolates grouped as having dual resistance, or possessing one of the five mutations conferring SDHI resistance in addition to the F129L mutation conveying QoI resistance, had mean spore germination percentages that were not significantly different from sensitive isolates (Fig. 3.1). The mean spore germination of dual-resistant isolates, as well as mean spore germination of isolates resistant to three chemical classes, were also not significantly different from isolates only possessing a single mutation conferring resistance to one chemical class (Fig. 3.1).

Table 3.3. Fitness parameters of grouped SDHI-resistant and -sensitive isolates of *Alternaria solani.*

	Fitness parameters[x]		
	In vitro		In vivo
Mutation	Spore Germination (%)[y]	Mycelial Expansion (mm)	Aggressiveness (RAUDPC)[z]
Sensitive	99 a	51.82 a	0.0584 b
H278Y	99 a	51.62 a	0.0585 b
H278R	99 a	51.60 a	0.0583 b
H134R	99 a	51.61 a	0.0586 b
H133R	99 a	51.64 a	0.0585 b
D123E	99 a	51.61 a	0.0712 a

[x] Mean values of groups followed by the same lowercase letter are not significantly different according to Fisher's protected least significant difference test at P = 0.05.
[y] Percentage of spores germination out of 50.
[z] RAUDPC = Relative area under the disease progress curve.

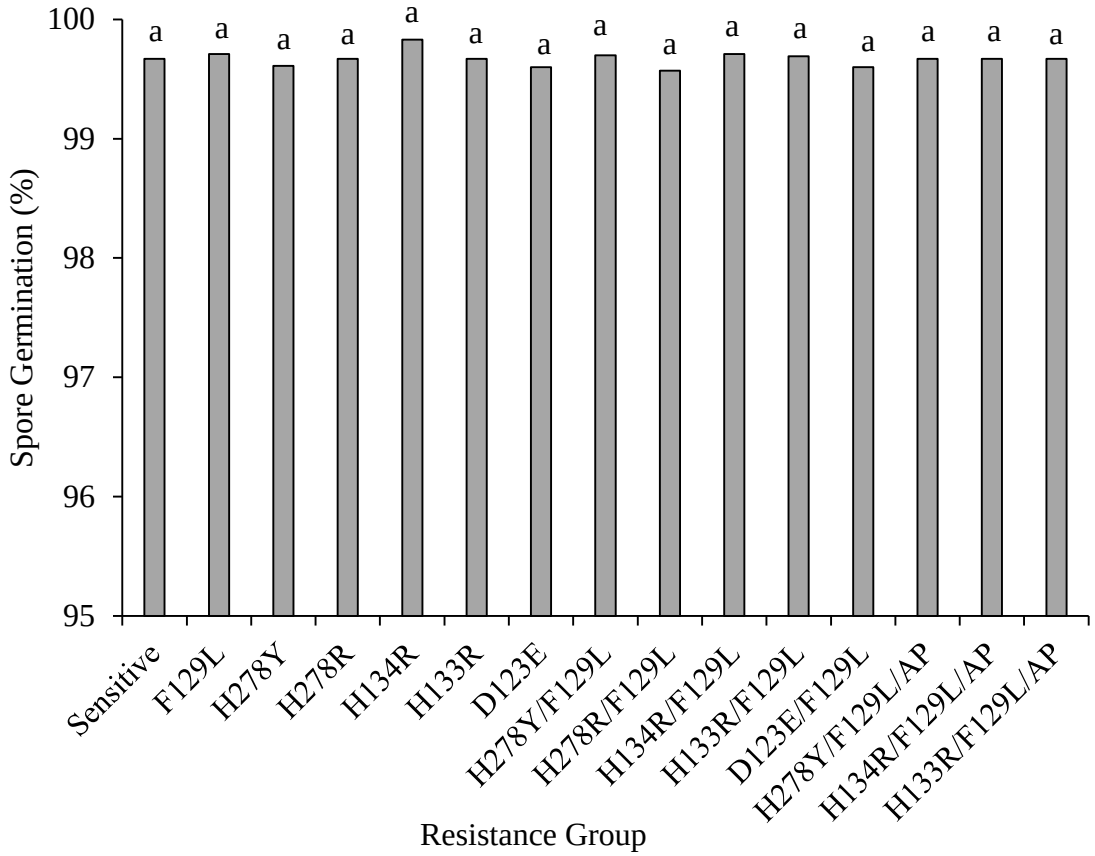

Fig. 3.1. Spore germination of grouped fungicide-resistant and -sensitive isolates of *Alternaria solani*. The H278Y, H278R, H134R, H133R, and D123E mutations are associated with SDHI resistance. The F129L mutation is associated with QoI resistance. AP denotes isolates found to have reduced sensitivity to pyrimethanil (Fonseka and Gudmestad, 2016). Groups with several denotations possess resistance to multiple chemical classes. Columns with the same letter are not significantly different according to Fisher's protected least significant difference test ($P < 0.05$).

Mycelial Expansion. Mean mycelial expansion among grouped SDHI-resistant and SDHI-sensitive isolates were not significantly different ($P = 0.77$) (Table 3.3). SDHI-sensitive isolates that did not possess any of the mutations associated with SDHI resistance were determined to have a mean mycelial expansion of 51.82 mm, which was higher, but not significantly, than SDHI-mutant isolate groups (H278Y, H278R, H134R, H133R, and D123E-mutants). There was also no significant difference in mean mycelial expansion among any of the SDHI-mutant groups, which ranged from 51.64 mm to 51.60 mm. (Table 3.3). When *A. solani* isolates were categorized into additional mutant groups to determine differences among isolates

possessing resistance to multiple chemical classes, there was also no significant difference in mycelial expansion (P = 0.51). Sensitive *A. solani* isolates, or isolates possessing no mutations associated with resistance to either QoIs or SDHIs had a mean mycelial expansion of 51.75 mm, which was not significantly different from the mean expansion of grouped isolates possessing one or two mutations (Fig. 3.2). The mean mycelial expansion of isolates with either the H278Y, H133R, or H134R in addition to the F129L mutation and reduced sensitivity to pyrimethanil were determined to have mean mycelial expansion values of 51.33 mm, 51.25 and 51.44 mm, respectively, which were not significantly different from grouped sensitive isolates or groups of isolates possessing resistance to one or two chemical classes (Fig. 3.2).

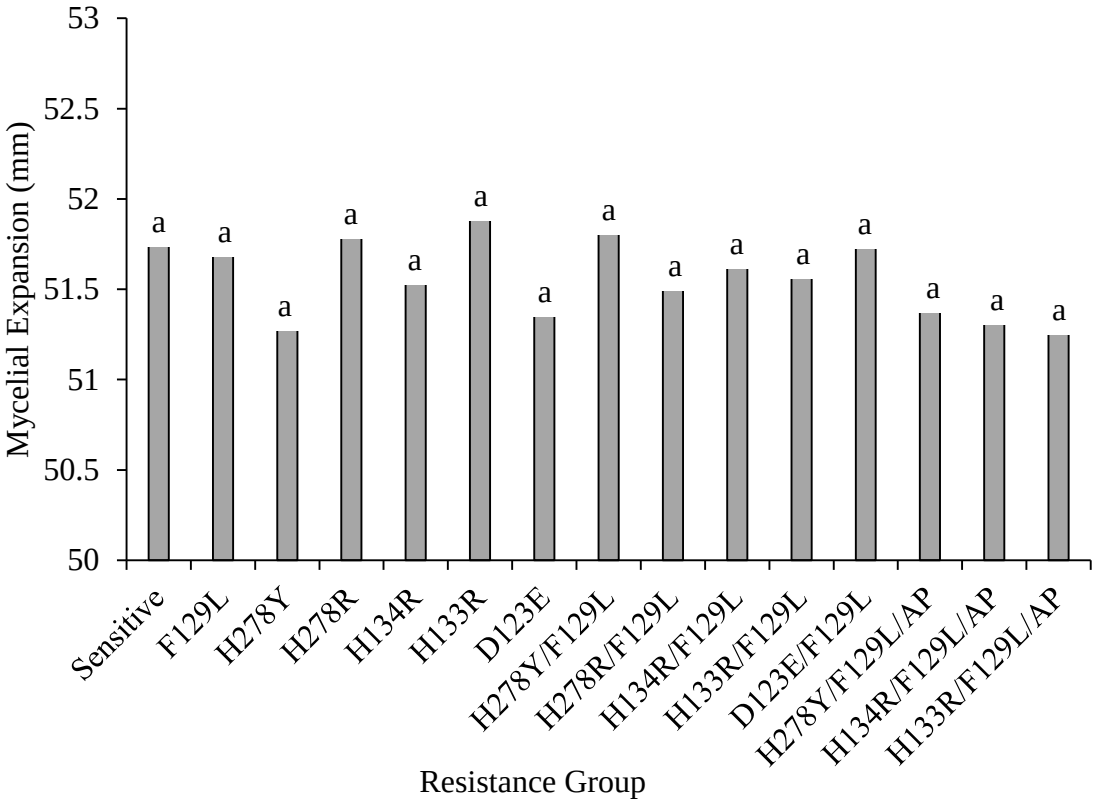

Fig. 3.2. Mycelial expansion of grouped fungicide-resistant and -sensitive isolates of *Alternaria solani*. The H278Y, H278R, H134R, H133R, and D123E mutations are associated with SDHI resistance. The F129L is associated with QoI resistance. AP denotes isolates found to have reduced sensitivity to pyrimethanil (Fonseka and Gudmestad, 2016). Groups with several denotations possess resistance to multiple chemical classes. Columns with the same letter are not significantly different according to Fisher's protected least significant difference test ($P < 0.05$).

Determination of realized fitness of fungicide-resistant and -sensitive *A. solani* isolates in vivo. *Aggressiveness*. All *A. solani* isolates evaluated for aggressiveness in greenhouse assays caused severe disease symptoms on tomato leaves. There were significant differences in mean RAUDPC values of SDHI-sensitive and -resistant isolates ($P = <0.0001$), with the 20 D123E-mutant isolates having a mean RAUDPC value of 0.0712, significantly higher than the 20 SDHI-sensitive isolates, with a mean RAUDPC value of 0.0584 (Table 3.3). *A. solani* isolates with the D123E mutation were also determined to have significantly higher RAUDPC values compared to other SDHI-mutant groups of H278Y, H278R, H134R, and

H133R-mutant isolates. H278R-mutant isolates were determined to have an RAUDPC value of 0.0583, which was the lowest of all grouped SDHI-resistant isolates, but this was not significantly different from SDHI-sensitive isolates or H278Y, H134R, and H133R-mutant isolates. Only the D123E mutation isolate group were found to have significantly higher aggressiveness in vivo compared to isolates with other SDHI mutations (Table 3.3; Fig. 3.3).

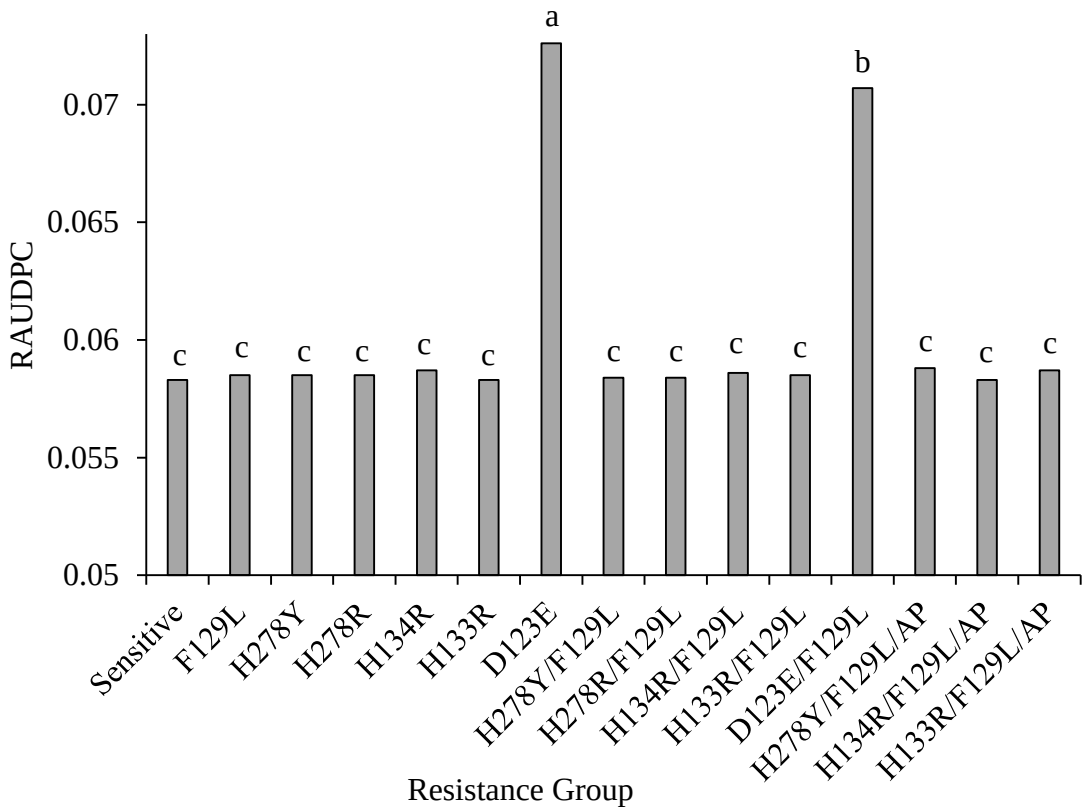

Fig. 3.3. Relative area under the disease progress curve (RAUDPC) values of grouped fungicide-resistant and -sensitive isolates of *Alternaria solani* as determined in greenhouse assays. The H278Y, H278R, H134R, H133R, and D123E mutations are associated with SDHI resistance. The F129L is associated with QoI resistance. AP denotes isolates found to have reduced sensitivity to pyrimethanil (Fonseka and Gudmestad, 2016). Groups with several denotations possess resistance to multiple chemical classes. Columns with the same letter are not significantly different according to Fisher's protected least significant difference test ($P < 0.05$).

When *A. solani* isolates were further categorized into sub-groups with multiple fungicide resistances, isolates possessing the D123E mutation were found also to have significantly higher

aggressiveness in vivo compared to other multiple fungicide-resistant isolate groups based on mean RAUDPC values (P = <0.0001) (Fig. 3.3). *A. solani* isolates possessing both the D123E mutation and the F129L mutation conveying dual resistance to SDHIs and QoI were determined to have significantly higher mean RAUDPC values compared to sensitive isolates and other mutant groups. D123E-mutants that did not have the F129L mutation were determined to have a mean RAUDPC value of 0.0726, which was significantly higher compared to a mean RAUDPC values of 0.0707 for D123E-mutants also possessing the F129L mutation (Fig. 3.3). However, RAUDPC values for isolates categorized into other fungicide mutation groups was not significantly different than the disease severity caused by wild type *A. solani* isolates. For example, the mean RAUDPC value for isolates possessing single SDHI mutations H278Y, H278R, H134R was not significantly different from isolates possessing these mutations and the F129L mutation (Fig. 3.3). Additionally, *A. solani* isolates possessing triple resistance to SDHIs, QoIs, and APs by virtue of possessing the H278Y, H134R, or H133R, the F129L mutation and reduced sensitivity to pyrimethanil had mean RAUDPC values of 0.0588, 0.0583, and 0.0587, respectively, which were not significantly different from the mean RAUDPC value of 0.0584 for wild type isolates (Fig. 3.3). All fungicide resistance groups, regardless of possessing resistance to one, two, or three fungicide chemical classes, were similar in in vivo aggressiveness compared to wild type isolates (Fig. 3.3).

Discussion

This is the first study evaluating parasitic fitness of SDHI-resistant *A. solani* isolates and isolates resistance to multiple chemical classes. The recent documentation of the widespread fungicide resistance in *A. solani* populations across the United States (Gudmestad et al. 2013; Fairchild et al. 2013; Mallik et al. 2014; Fonseka and Gudmestad et al. 2016; Tymon and

Johnson, 2014) necessitates the implementation of fungicide resistance management strategies that can functionally delay further resistance development. To successfully implement these strategies and to determine how effective they will be at delaying the selection of resistant strains, knowledge of parasitic fitness is critical because resistance may be accompanied by fitness penalties that influence the risk of resistance build-up in the absence of selection pressure (Mikaberidze et al. 2014; Milgroom et al. 1989). Thus, the characterization of resistant isolates in terms of fitness may allow us to predict the behavior of the pathogen population (Avenot and Michailides, 2010). In the current study, parasitic fitness was evaluated both in terms of "predicted fitness" (measurement of parameters in vitro) and "realized fitness" (evaluation of fungicide-resistant and -sensitive isolates in vivo) (Antonovics and Alexander, 1989).

SDHI-sensitive and -resistant *A. solani* isolates in this study were shown to have no significant differences in in vitro spore germination or mycelial expansion, suggesting the lack of any fitness penalty under these parameters in vitro. Similarly, many other studies evaluating fungicide-resistant isolates of several pathogens have identified no differences in predicted fitness parameters (Corio-Costet et al. 2010; Peever and Milgroom, 1994; Billard et al. 2012; Kim and Xiao, 2011). For example, boscalid-resistant and -sensitive isolates of *A. alternata* of pistachio were evaluated and revealed to have no significant differences in predicted fitness components of spore germination, hyphal growth, or sporulation, and following successive subculturing on non-fungicide amended potato dextrose agar, resistant isolates were stable after multiple generations in the absence of selection pressure (Avenot and Michailides, 2007). Similarly, in other pathogens, such as *D. bryoniae* (Stevenson et al. 2008) and *C. cassiicola* (Miyamoto et al. 2009), no differences in the mycelial growth rates were observed between boscalid-resistant and -sensitive field isolates on non-amended media. A previous study with *A.*

solani evaluated in vitro and in vivo fitness of isolates possessing the F129L mutation conferring QoI resistance and found that F129L-mutants, compared to sensitive isolates, had significantly lower spore germination in vitro but higher aggressiveness in vivo (Pasche and Gudmestad, 2008). This suggests the lack of spore germination is not a significant fitness penalty causing reduced competition of F129L-mutants in the field. A few studies related to the fitness and competitive ability of QoI laboratory-induced mutants of *Cercospora beticola* and *Botrytis cinerea* have identified fitness penalties (Malandrakis et al. 2006; Markoglou et al. 2006), but it is likely that this was due to pleiotropic effects of several accumulated mutations induced by mutagenesis during experimentation (Karaoglanidis and Michailides, 2011). Another study evaluating laboratory-induced boscalid-resistant mutants of *Penicillium expansum* found no difference between resistant mutants and wild type isolates in a number of in vitro fitness parameters including osmotic sensitivity, spore production, and spore germination (Malandrakis et al. 2017). However, most *P. expansum* laboratory induced mutants did show a significant reduction in mycelial growth compared to wild type isolates, but also demonstrated an increase in mycotoxin production relative to wild type isolates (Malandrakis et al. 2017). These data suggest the existence of fitness penalties in SDHI- or QoI-resistant isolates that are not just pathogen dependent but may also depend upon fitness component evaluated.

Perhaps the most interesting finding of these studies is that *A. solani* isolates possessing the D123E mutation on the *AsSdhD* gene conferring SDHI resistance were found to have significantly higher aggressiveness in vivo compared to wild type isolates, as well as isolates possessing other mutations conferring SDHI resistance. This may explain why isolates possessing the D123E mutation, which conveys a high level of resistance to the SDHI fungicides boscalid and penthiopyrad (Mallik et al. 2011), were collected at increasingly higher frequencies

throughout a multi-year spatial survey by our research group (Bauske et al. Plant Disease in review). All D123E-mutant *A. solani* isolates, regardless of the presence of resistance to QoI or AP fungicides, were shown to have significantly higher aggressiveness in vivo compared to wild type isolates. This suggests the absence of any fitness penalty associated with the accumulation of other fungicide resistance. Other studies have also suggested a fitness advantage of SDHI-resistant isolates, such as with boscalid-resistant isolates of *A. alternata* in pistachio, which were recovered from plant tissue at high proportions before any application of fungicides during the growing season (Avenot and Michailides, 2008). An increase of fitness was also demonstrated in *B. cinerea* isolates possessing the H272R mutation in the *BcSdhB* gene and H272R-mutants were also recovered at high frequency within SDHI-resistant populations of *B. cinerea* (Veloukas et al. 2014; Fernandez-Ortuno et al. 2012; Kim and Xiao, 2010). Other studies have found that *A. alternata* isolates from peach that possess the H277Y or H134R mutations conferring SDHI-resistance did not suffer any obvious fitness penalties and that H277Y-mutant *A. alternata* isolates were found to have greater fitness than boscalid-sensitive isolates (Fan et al. 2015).

This study also found no differences in spore germination or mycelial expansion between sensitive isolates and *A. solani* isolates possessing resistance to several chemical classes. While this is the first study evaluating the fitness of *A. solani* isolates resistant to multiple chemical classes, previous studies have identified or refuted fitness costs attributed to dual fungicide resistance in other pathogens. In *B. cinerea* isolates with dual resistance to QoIs and SDHIs, isolates possessing the G143A mutation conveying a high level of resistance to QoIs and different *SdhB* mutations were shown to suffer significant fitness costs, including lower values for multiple fitness components and the dominance of sensitive *B. cinerea* isolates in competition

experiments (Veloukas et al. 2014). Other fitness and competitive penalties have been identified in *B. cineara* isolates resistant to five or more chemical classes (Chen et al. 2016). In contrast, other studies have reported that *B cinerea* isolates collected from apple with dual resistance to SDHIs and QoIs expressed fitness similar to that of sensitive isolates (Kim and Xiao, 2011). However, *B. cinerea* isolates possessing multiple mutations in the *BcSdhB* gene conferring resistance to SDHIs demonstrated a high fitness cost compared with wild-type strains (Laleve et al. 2013), suggesting fitness penalties attributed to the accumulation of SDHI mutations in the same isolate. Evaluation of *A. alternata* isolates resistant to SDHIs, QoIs, and methyl benzimidazoles (MBCs) has found both fitness costs and higher fitness of resistant isolates depending on mutation and fitness parameter (Fan et al. 2015). Furthermore, *A. alternata* isolates possessing the D123E mutation had hypersensitivity to oxidative stress and weak sporulation. Although the current study suggests higher fitness of D123E-mutant *A. solani* isolates, the same mutation may or may not be associated with fitness penalties in different pathogens (Karaoglanidis et al. 2011; Rallos et al. 2014). Nothing is known about additional genetic variation or genetic background of *A. solani* isolates possessing the D123E mutation and further research utilizing whole-genome sequencing to associate polymorphisms with reduced sensitivity to fungicides or fitness may be necessary.

The absence of obvious fitness penalties in *A. solani* isolates resistant to a single or multiple chemical classes in this study have important implications for the management of early blight of potato. The research reported here clearly indicates that isolates of *A. solani* resistant to SDHI, QoI, and/or AP fungicides have similar in vitro and in vivo parasitic fitness compared to sensitive isolates, which suggests that resistant isolates may successfully compete with sensitive isolates under field conditions. These data also substantiate the previous findings that *A. solani*

isolates possessing the F129L mutations do not suffer from substantial fitness penalties. Additional studies are needed to further elucidate the competitive fitness of fungicide-resistant isolates of *A. solani* in the presence and absence of fungicide selection pressure. Future research will focus on competition experiments using mixed inocula of isolates with equal aggressiveness at various ratios. Inoculation experiments in vivo using mixtures of sensitive *A. solani* isolates and isolates possessing mutations conferring fungicide resistance, and the determination of their recovery ratios from symptomatic plants, will provide an additional assessment of competitive fitness by more closely simulating an agricultural environment. However, this study reasonably predicts using the current evaluation parameters, considering the absence of any fitness penalties under these parameters, the frequency of SDHI-, QoI-, and AP-resistant *A. solani* isolates will be maintained in the pathogen population in the absence of selection pressure and lead to a continual decline in the performance of certain chemistries in these classes.

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GENERAL CONCLUSIONS

Early blight of potato (*Solanum tuberosum*), caused by *Alternaria solani* Sorauer, is a chronic foliar disease of potato present every growing season, and is particularly problematic in the midwestern portion of the United States (Gudmestad et al. 2013). The disease is characterized by small, dark brown to black, discrete lesions with a concentric ring pattern that tend to be evident on senescing leaf tissue (Franc and Christ, 2001). Severe foliar infection during the tuber bulking stage can result in the formation of small tubers and reduced yield. While yield losses have been shown to reach above 30%, they rarely exceed 20%, and intensive fungicide treatment throughout the growing season minimizes losses to less than 5% (Pscheidt and Stevenson, 1988). However, *A. solani* may also cause a dry rot on tubers which further reduces the quantity and quality of marketable tubers (Nnodu et al. 1982). Since most commercially acceptable potato cultivars are susceptible to some degree, the timely application of foliar fungicides is the primary tactic used to manage the disease (Gudmestad et al. 2013).

A wide range of foliar fungicide chemistries can be employed to manage early blight in potato. The application of standard protectants such as chlorothalonil and mancozeb in fungicide regimes, beginning early in the growing season and continuing until vine desiccation, is an integral part of chemical control measures used to manage the disease (Pasche et al. 2004, Pscheidt and Stevenson, 1988). However, the frequently used standard protectants do not provide sufficient control under high disease pressure. As a result, the application of more expensive single-site mode of action chemistries of the quinone outside inhibiting (QoI), succinate dehydrogenase inhibiting (SDHI), and anilino-pyrimidine (AP) classes are necessary to achieve adequate control (Pasche et al. 2004, 2005; Gudmestad et al. 2013; Mallik et al. 2014; Fonseka and Gudmestad, 2016; Yellareddygari et al. 2016).

The QoI fungicides were first introduced in 1999, but reduced efficacy was reported within two years due to intensive selection pressure caused by extensive usage in production systems (Pasche et al. 2004). QoI resistance due to the F129L mutation in the cyctochrome b gene was first detected in North Dakota and Nebraska in 2001 (Pasche et al. 2004, 2005) and determined to be widespread throughout the United States by 2006 (Pasche and Gudmestad, 2008). Boscalid, the first of the newer generation SDHIs, was registered for use on potato in 2005 and provided excellent control, replacing QoI fungicides in the foliar fungicide program. However, resistance to boscalid was detected in Idaho in 2009 (Wharton et al. 2012) and determined to be widespread throughout multiple states by 2012 (Gudmestad et al. 2013; Mallik et al. 2014). Resistance to SDHIs in *A. solani* is independently conveyed by five different point mutations on three *AsSdh* genes.

There were several objectives in this research, including i) determine the spatial and temporal frequency distribution of mutations conferring QoI and SDHI resistance across the United States in a large and diverse population of *A. solani*; ii) determine the effect of fluopyram on the frequency of the D123E mutation in *A. solani*; and iii) determine the predicted and realized parasitic fitness of fungicide-sensitive and -resistant *A. solani* isolates and characterize fitness penalties in isolates resistant to one, two, or three chemical classes (QoIs, SDHIs, and APs).

A total of 1,323 *A. solani* isolates were recovered from 11 states from 2013 through 2015 to determine the distribution of mutations associated with SDHI and QoI resistance. Results were compared to a previously characterized collection of *A. solani* isolates recovered across six states from 2010 through 2011. Previously, it was demonstrated that isolates possessing mutations on the *AsSdhB* gene were the most prevalent and generally distributed among six states, constituting

67% of the *A. solani* population in 2011 (Mallik et al. 2014). *A. solani* isolates possessing mutations on the *AsSdhC* or *AsSdhD* genes constituted 24% of the population in 2011 and were only detected in specific regions (Mallik et al. 2014). However, *A. solani* isolates possessing the H134R mutation on the *AsSdhC* gene were the most prevalent from 2013 through 2015, constituting 36% of the population. Most importantly, *A. solani* isolates possessing the D123E mutation, comprising just 1.5% of isolates collected in 2011, consisted of 12% of the isolates recovered in 2015.

Experiments in laboratory, greenhouse, and field settings were conducted across multiple years to determine the impact of SDHI fungicides on the frequency of *A. solani* isolates possessing the D123E mutation. A total of 118 isolates with the D123E mutation were evaluated for boscalid and fluopyram sensitivity in vitro and it was determined that isolates possessing the D123E mutation were inhibited by less than 5 parts per million of fluopyram, demonstrating sensitivity in vitro. However, when 15 *A. solani* isolates with similar EC_{50} values in vitro were evaluated for percentage disease control provided by boscalid and fluopyram in vivo, there were significant differences in disease control among isolates. Isolates with the D123E mutation exhibited significantly reduced disease control under the application of fluopyram compared to SDHI-sensitive isolates and SDHI mutant isolates possessing the H278R, H134R, or H133R mutations. Interestingly, isolates possessing the D123E mutation were recovered at significantly higher frequencies from treatments where fluopyram was applied in-furrow at planting in the field. However, in treatments receiving the biological control Serenade Soil™ (*Bacillus subtilis*) in addition to fluopyram in-furrow at planting, *A. solani* isolates with the D123E mutation were recovered at significantly lower frequencies compared to when fluopyram was applied alone in-furrow at planting. These data suggest *A. solani* isolates possessing the D123E mutation have a

selection advantage under a selection pressure from fluopyram compared to wild type isolates and other SDHI-resistant isolates possessing the H278R, H134R, or H133R mutations.

Finally, the predicted and realized fitness of 120 fungicide-resistant and -sensitive *A. solani* isolates was evaluated using the parameters of spore germination and mycelial expansion in vitro and aggressiveness in vivo. There were no significant differences in mycelial expansion or spore germination in vitro among wild type *A. solani* isolates and isolates resistant to one, two, or three chemical classes (SDHIs, QoIs, and APs). Only isolates with the D123E mutation were demonstrated to have significantly higher aggressiveness in vivo based on relative area under the disease progress curve (RAUDPC) values compared to wild type *A. solani* isolates. These results demonstrate the absence of any fitness penalties in *A. solani* isolates resistant to one, two, or three chemical classes based on these parameters, and suggest that fungicide-resistant isolates of *A. solani* may compete successfully with wild type isolates under field conditions.

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APPENDIX A. REPRODUCIBILITY OF IN VITRO FITNESS PARAMETERS FOR GROUPED FUNGICIDE-RESISTANT AND -SENSITIVE ISOLATES OF *ALTERNARIA SOLANI*

Table A.1. Reproducibility of spore germination assays for determining in vitro parasitic fitness of grouped fungicide-resistant and -sensitive isolates of *Alternaria solani*.

Isolate	Mean Spore Germination (%)	95% Confidence Interval	Coefficient of Variance
13-1	99	(99-100)	0.02
526-3	99	(99-100)	0.01

Table A.2. Reproducibility of mycelial expansion assays for determining in vitro parasitic fitness of grouped fungicide-resistant and -sensitive isolates of *Alternaria solani*.

Isolate	Mean Mycelial Expansion (mm)	95% Confidence Interval	Coefficient of Variance
13-1	51.25	(51.67-52.25)	0.01
526-3	51.33	(51.51-52.75)	0.01

APPENDIX B. SUMMARY OF STATISTICAL ANALYSIS FOR IN VITRO FITNESS PARAMETERS

Table B.1. Analysis of variance for spore germination among grouped SDHI-resistant and -sensitive isolates of *Alternaria solani* as determined in in vitro assays.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
SDHI mutation groups	5	0.5433	0.96	0.4441
Trial	1	0.0055	0.01	0.9213

Table B.2. Analysis of variance for spore germination among grouped fungicide-resistant and -sensitive isolates of *Alternaria solani* as determined in in vitro assays.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Resistance groups	14	0.1796	0.32	0.9921
Trial	1	0.0055	0.01	0.9213

Table B.3. Analysis of variance for mycelial expansion among grouped SDHI-resistant and -sensitive isolates of *Alternaria solani* as determined in in vitro assays.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
SDHI mutation groups	5	0.8380	0.61	0.6885
Trial	1	0.1125	0.08	0.7740

Table B.4. Analysis of variance for mycelial expansion among grouped fungicide-resistant and -sensitive isolates of *Alternaria solani* as determined in in vitro assays.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Resistance groups	14	1.2051	0.94	0.5159
Trial	1	0.0680	0.05	0.8179

APPENDIX C. REPRODUCIBILITY OF IN VITRO SENSITIVITY ASSAYS FOR *ALTERNARIA SOLANI* ISOLATES POSSESSING THE D123E MUTATION

Table C.1. Reproducibility of in vitro assays for determining boscalid sensitivity of *Alternaria solani* isolates possessing the D123E mutation.

Isolate	Mean EC_{50}	95% Confidence Interval	Coefficient of Variance
13-1	0.37	(0.27-0.50)	0.03
526-3	0.50	(0.40-0.63)	0.01

Table C.2. Reproducibility of in vitro assays for determining fluopyram sensitivity of *Alternaria solani* isolates possessing the D123E mutation.

Isolate	Mean EC_{50}	95% Confidence Interval	Coefficient of Variance
13-1	0.20	(0.14-0.25)	0.01
526-3	0.33	(0.27-0.45)	0.01

APPENDIX D. SUMMARY OF STATISTICAL ANALYSIS FOR IN VITRO SENSITIVITY TESTS FOR BOSCALID AND FLUOPYRAM

Table D.1. Combined analysis of variance for in vitro sensitivity of *Alternaria solani* isolates possessing the D123E mutation to boscalid.

Source of variation	**Degrees of freedom**	**Mean squares**	**F value**	***P* value**
Experiment	1	0.0175	0.67	0.4294
Isolate	117	0.3201	12.26	<0.0001
Experiment × Isolate	117	0.0219	0.84	0.0541
Error	236	0.0261		
Corrected Total				

Table D.2. Combined analysis of variance for in vitro sensitivity of *Alternaria solani* isolates possessing the D123E mutation to fluopyram.

Source of variation	**Degrees of freedom**	**Mean squares**	**F value**	***P* value**
Experiment	1	0.0002	0.70	0.8536
Isolate	117	0.0128	42.58	<0.0001
Experiment × Isolate	117	0.0004	1.53	0.2757
Error	236	0.0003		
Corrected Total				

APPENDIX E. SUMMARY OF STATISTICAL ANALYSIS FOR IN VIVO SENSITIVITY TESTS FOR BOSCALID AND FLUOPYRAM

Table E.1. Combined analysis of variance of in vivo percentage disease control of *Alternaria solani isolates* provided by boscalid.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Experiment	1	1.7182	3.40	0.0664
Rep	2	0.8231	1.63	0.1983
Isolate	14	1755.3655	3471.76	<0.0001
Rep × Isolate	28	0.6551	1.30	0.1518
Fungicide Concentration	3	91761.0145	181485	<0.0001
Isolate × Fungicide Concentration	42	181.5842	359.14	<0.0001
Error	269	0.5056		
Total	359			

Table E.2. Combined analysis of variance of in vivo percentage disease control of *Alternaria solani* isolates provided by fluopyram.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Experiment	1	8.1332	0.77	0.3802
Rep	2	17.6921	1.68	0.1882
Isolate	14	4374.1829	415.54	<0.0001
Rep × Isolate	28	4.6272	0.44	0.9945
Fungicide Concentration	3	93569.3789	8888.88	<0.0001
Isolate × Fungicide Concentration	42	504.5303	47.93	<0.0001
Error	269	10.5266		
Total	359			