SPATIAL AND TEMPORAL SENSITIVITY OF *ALTERNARIA* SPECIES ASSOCIATED WITH POTATO FOLIAR DISEASES TO DEMETHYLATION INHIBITING AND ANILINO-PYRIMIDINE FUNGICIDES

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ABSTRACT

Early blight and brown spot, caused by *Alternaria solani* and *Alternaria alternata*, respectively, are important foliar diseases of potato, affecting both tuber yield and quality. Most of the commercial cultivars lack resistance; therefore, application of foliar fungicides remains a primary management strategy. Correlation coefficients comparing EC_{50} values for conidial germination and mycelial growth of *A. solani* and *A. alternata* in response to boscalid and fluopyram, respectively, were low, indicating that the association between these two *in vitro* assays was very weak. Baseline sensitivities of *Alternaria* spp. to difenoconazole, metconazole, and pyrimethanil using mycelial growth assays demonstrated high intrinsic activity against the two pathogens. Six out of 245 *A. solani* isolates exhibited reduced-sensitivity to pyrimethanil in *in vitro* assays and reduced-sensitive isolates were not controlled except at 100 µg/ml in greenhouse efficacy tests. The DMI chemistries and pyrimethanil remain valuable options for fungicide rotation programs in areas of high disease pressure.

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TABLE OF CONTENTS

ABSTRACT....................iii

ACKNOWLEDGEMENTS iv

LIST OF TABLES.................... vii

LIST OF FIGURES viii

LIST OF APPENDIX TABLES ix

LITERATURE REVIEW 1

History of Potato 1

Taxonomy and Nomenclature....................1

Biology of *Alternaria solani* and *Alternaria alternata* 6

Symptomatology 13

Epidemiology....................14

Disease Management 17

Fungicide Resistance 28

MATERIALS AND METHODS 40

Collection and Maintenance of Isolates 40

Comparison of Conidial Germination Inhibition to Mycelial Growth Inhibition 41

In Vitro Baseline Sensitivity of *Alternaria solani* and *Alternaria alternata* to Difenoconazole and Metconazole 46

In Vitro Baseline Sensitivity of *Alternaria solani* and *Alternaria alternata* to Pyrimethanil 47

Sensitivity of Non-Baseline Isolates of *Alternaria solani* to Difenoconazole, Metconazole, and Pyrimethanil 47

Effect of Reduced-Sensitivity of *Alternaria solani* to Pyrimethanil on Disease Control .47

Statistical Analysis....................49

RESULTS....................51

Comparison of Conidial Germination Inhibition to Mycelial Growth Inhibition 51

In vitro Baseline Sensitivity of *Alternaria solani* and *Alternaria alternata* to Difenoconazole and Metconazole 55

In Vitro Baseline Sensitivity of *Alternaria solani* and *Alternaria alternata* to Pyrimethanil 59

Sensitivity of Non-Baseline isolates of *Alternaria solani* to Difenoconazole, Metconazole, and Pyrimethanil 61

Effect of Reduced-Sensitivity of *Alternaria solani* to Pyrimethanil on Disease Control..64

DISCUSSION 66

LITERATURE CITED..75

APPENDIX A. COLLECTION DATA OF *ALTERNARIA SOLANI* ISOLATES COLLECTED FROM FIELDS IN THE 2010, 2011, 2012, 2013, AND 2014 SEASONS FROM SEVERAL STATES 92

APPENDIX B. COLLECTION DATA OF *ALTERNARIA ALTERNATA* ISOLATES COLLECTED FROM FIELDS IN THE 2011, 2013, AND 2014 SEASONS FROM SEVERAL STATES...99

APPENDIX C. CV-8 AGAR MEDIUM 102

APPENDIX D. L-ASPARAGINE (ASP-AGAR) MEDIUM..103

APPENDIX E. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* COMPARISON OF CONIDIAL GERMINATION TO MYCELIAL GROWTH (FLUOPYRAM)...104

APPENDIX F. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* COMPARISON OF CONIDIAL GERMINATION TO MYCELIAL GROWTH (BOSCALID) 105

APPENDIX G. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* BASELINE SENSITIVITY TESTS OF *ALTERNARIA SOLANI* TO DMI FUNGICIDES........106

APPENDIX H. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* BASELINE SENSITIVITY TESTS OF *ALTERNARIA ALTERNATA* TO DMI FUNGICIDES...107

APPENDIX I. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* BASELINE SENSITIVITY TESTS OF PYRIMETHANIL...108

APPENDIX J. SUMMARY OF STATISTICAL ANALYSIS FOR PYRIMETHANIL IN VIVO SENSITIVITY TESTS 109

LIST OF TABLES

Table Page

1. Collection information and mean *in vitro* sensitivity of *Alternaria alternata* to fluopyram 42

2. Collection information and mean *in vitro* sensitivity of *Alternaria solani* to boscalid 45

3. *Alternaria solani* isolates tested *in vivo* for sensitivity to pyrimethanil 48

4. Range, mean, median EC_{50} values, and coefficient of variability based on log_{10}-transformed EC_{50} values of isolates of *Alternaria alternata* for each *in vitro* method in determining fluopyram sensitivity 52

5. Range, mean, and median EC_{50} values based on log_{10}-transformed EC_{50} values of isolates of *Alternaria solani* for each *in vitro* method in determining boscalid sensitivity 54

6. Reproducibility of the *in vitro* assays for determining boscalid sensitivity of isolates of *Alternaria solani* 54

7. Range, mean, and median EC_{50} values based on log_{10}-transformed EC_{50} values of baseline isolates of *Alternaria solani* to DMI fungicides 55

8. Reproducibility of the *in vitro* assays for determining DMI sensitivity of baseline isolates of *Alternaria solani* 56

9. Range, mean, median EC_{50} values, and coefficient of variability based on log_{10}-transformed EC_{50} values of baseline isolates of *Alternaria alternata* to DMI fungicides 58

10. Range, mean, median EC_{50} values, and coefficient of variability based on log_{10}-transformed EC_{50} values of baseline isolates of *Alternaria* spp. isolates to pyrimethanil 60

11. Reproducibility of the *in vitro* assays for determining pyrimethanil sensitivity of baseline isolates of *Alternaria solani* 61

12. Mean *in vivo* percentage disease control of *Alternaria solani* isolates by pyrimethanil as determined in greenhouse assays 65

LIST OF FIGURES

Figure Page

1. Scatter diagram of the linear correlation between conidial germination inhibition and mycelial growth inhibition of *Alternaria alternata* in response to fluopyram 52

2. Scatter diagram of the linear correlation between conidial germination inhibition and mycelial growth inhibition of *Alternaria solani* in response to boscalid...........................54

3. Frequency distribution of sensitivity of 57 baseline *Alternaria solani* isolates to DMI fungicides 56

4. Scatter diagram of the linear correlation between the *in vitro* difenoconazole and metconazole sensitivity of 57 baseline *Alternaria solani* isolates 57

5. Frequency distribution of sensitivity of 50 baseline *Alternaria alternata* isolates to DMI fungicides 58

6. Scatter diagram of the linear correlation between the *in vitro* difenoconazole and metconazole sensitivity of 50 baseline *Alternaria alternata* isolates 59

7. Frequency distribution of sensitivity of baseline *Alternaria* spp. isolates to pyrimethanil 60

8. Mean EC_{50} values for *in vitro* isolate sensitivity of *Alternaria solani* to DMI fungicides across years 62

9. Scatter diagram of the linear correlation between the *in vitro* difenoconazole and metconazole sensitivity of 245 *Alternaria solani* isolates collected from 2010 to 2014...63

10. Mean EC_{50} values for *in vitro* isolate sensitivity of *Alternaria solani* and *Alternaria alternata* to pyrimethanil across years 64

11. Mean *in vivo* percentage disease control of *Alternaria solani* isolates by pyrimethanil as determined in greenhouse assays 65

LIST OF APPENDIX TABLES

Table	Page
E1. Combined analysis of variance for *in vitro* sensitivity of *Alternaria alternata* to fluopyram based on conidial germination inhibition	104
E2. Combined analysis of variance for *in vitro* sensitivity of *Alternaria alternata* to fluopyram based on mycelial growth inhibition	104
E3. Summary of one-way analysis of variance comparing conidial germination inhibition and mycelial growth inhibition of *Alternaria alternata* in response to fluopyram	104
F1. Combined analysis of variance for *in vitro* sensitivity of *Alternaria solani* to boscalid based on conidial germination inhibition	105
F2. Combined analysis of variance for *in vitro* sensitivity of *Alternaria solani* to boscalid based on mycelial inhibition	105
F3. Summary of one-way analysis of variance comparing conidial germination inhibition and mycelial growth inhibition of *Alternaria solani* in response to boscalid	105
G1. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria solani* to difenoconazole	106
G2. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria solani* to metconazole	106
H1. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria alternata* to difenoconazole	107
H2. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria alternata* to metconazole	107
I1. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria solani* to pyrimethanil	108
I2. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria alternata* to pyrimethanil	108
J1. Combined analysis of variance of *in vivo* percentage disease control for sensitive and reduced-sensitive *Alternaria solani* isolates to pyrimethanil	109

LITERATURE REVIEW

History of Potato

The genus *Solanum* is one of the largest angiosperm genera with approximately 1500 species, including important economical plants such as the potato, tomato, and eggplant, as well as other ornamental and medicinal plants (Bohs, 2007). The story of the potato (*Solanum tuberosum* L.), a herbaceous annual plant, begins about 10,000 years ago in the Andes of southern Peru (Ames and Spooner, 2008). The first report of cultivated potato outside of South America was in the Canary Islands, off northwest Africa in 1567 (Rios, 2007). A versatile, carbohydrate-rich tuber is the world's fifth largest food crop, following sugar cane, maize, rice, and wheat. The United States is the fifth largest potato producer with more than 19 million metric tons in 2013 (FAO, 2015). North Dakota is a leading potato producer in the United States with approximately 32,000 hectares of area harvested, following Idaho and Washington (USDA, 2015).

Late blight, Verticillium wilt, pink rot, early blight, and brown spot are common potato diseases that occur predominantly in the potato-producing regions in the United States. Potato early blight and brown spot are economically important foliar diseases of potato and cause premature defoliation of potato plants.

Taxonomy and Nomenclature

The genus *Alternaria* is classified in the Kingdom *Fungi*, Phylum *Ascomycota*, Family *Pleosporaceae*, and Order *Pleosporales* (Agrios, 1997). The genus *Alternaria* contains the most diverse and common forms of Ascomycota fungi, including aggressive and opportunistic plant pathogens or saprophytes on organic substances (Weir, 1998). The distinction between the behaviors is not quite established, because some species maintain an intermediate position and

shift from being a saprophyte to a parasite when they come across a weakened host (Rotem, 1994). *A. solani* Sorauer has a worldwide distribution due to the distribution and production of potato and tomato, while *A. alternata* (Fr.) Keissler affects Solanaceous plants and a variety of crops such as mango (Prusky, 1983), citrus (Solel, 1991), pistachio (Pryor and Michailides, 2002), banana (Parkunanet et al., 2013), date palm (Palou et al., 2013), tea (Zhou and Xu, 2014), and apple (Jurick el al., 2014).

The scientific classification of the genus *Alternaria* has been disputed since the early 1800s. The genus *Alternaria* was established in the early 19th century and *A. tenuis* (currently *A. alternata*) was the only species described (Nees, 1817). Although this classification was incomplete and in some aspects inaccurate, it was sufficient enough to describe and recognize the genus as *Alternaria* (Elliott, 1917). The genus *Macrosporium* was described by a similar classification publication (Fries, 1825) and was differentiated from *Cladosporium*, *Helminthosporium*, and *Sporodesmium*, but did not recognize the genus *Alternaria* (Tweedy and Powell, 1963). Although *Alternaria*-like specimens were placed under the genus Torula, the position was questioned. The existence of the genus *Alternaria* was later acknowledged and found that it differed from *Macrosporium* (Tweedy and Powell, 1963).

The generic characteristics and the form of the conidia (obclavate, pointed, and often having beaks) were defined as important features of the classification system of *Alternaria* spp. (Elliot, 1917). The publication considered *Alternaria* and *Macrosporium* to be two different groups and suggested that the genus *Alternaria* be divided into "groups of species" having similar spores. As the two genera appear to be similar, other researchers proposed *Macrosporium* to designate both groups (Angell, 1929). The classification dispute was resolved later in an

extensive study, which concluded *Alternaria* and *Macrosporium* were congeneric (Wilshire, 1933).

Early work on *Alternaria* considered that *Pleospora herbarum* (Pers.) Rabenh was the ascogenous stage of *Alternaria* (Ellis, 1971). But, pure cultures of *P. herbarum* did not produce the characteristic conidia of *Alternaria*, and pure *Alternaria* isolates did not produce any ascogenous stages (Tweedy and Powell, 1963). Furthermore, specific taxa of *Stemphylium* and *Ulocladium* that resemble *Alternaria* were misidentified as *Alternaria* spp. (Rotem, 1994). However, these two genera can be differentiated from *Alternaria* because they never form chains of conidia, and they produce spores without beaks. Descriptions by three research projects provided useful information distinguishing *Alternaria* spp. from the two genera (Ellis 1971; 1976; Neergaard, 1945). The binomials used for *Alternaria* spp. differ in various publications: *A. solani* Sorauer (Ellis, 1971), *A. porri* (Ell.) Neerg. f. sp. *solani* E. and M. (Neergaard, 1945), and *A. dauci* f. sp. *solani* (E. and M.) Neerg. (Joly, 1967).

Alternaria spp. are readily identified by the morphology of their large conidia. The conidia are catenate or solitary, typically ovoid or obclavate, often rostrate (beaked), pale to dark brown, and multicelled with transverse and frequently oblique or longitudinal septa (Ellis, 1971). The genus *Alternaria* was allocated into three groups based on catenulation: Longicatenatae, Brevicatenatae, and Noncatenatae (Neergaard, 1945). Longicatenatae includes the species that produce conidia in chains of approximately 10 spores or more, either beakless or very short beaks, as in *A. alternata.* Brevicatenatae group includes species that produce conidia in chains of with three to five spores with relatively short or relatively long beaks, as in *A. tenuisssima*. Noncatenatae species typically form conidia and may be beakless, as in *A. helianthi*, but usually have long beaks, as in *A. solani.* Some species differ from the assigned groups due to variability

in catenulation. For instance, some species in the Longicatenatae group may have isolates that produce solitary spores while some Noncatenatae species form short chains of two spores. Growth conditions also can influence catenulation on artificial medium. *A. brassicae* may form short chains, but it rarely forms chains on host plants (Rotem, 1994).

Apart from catenulation, there are various other characteristics used in the classification system. Other criteria include septation and size of the conidia, character of the conidial beak, the dimensions of the conidiophores, the host range of pathogenic species, and specific physiological properties (Neergaard, 1945). The dimensions of the spore body, including the beak, are considered the most essential features of a given species. The spores of most of the species are similar in terms of width, but vary in length (Rotem, 1994). Although, spore length can be used to differentiate species with distinct dimensions, in some cases, the spore lengths of different species overlap, thereby making identification difficult in some species in the *Alternaria* genus. Environmental factors such as substrate, light intensity, and temperature affect the variability of the spore dimensions. Variability within a species is often found in measurements of many isolates. Forty two isolates of *A. solani* were measured with average length of the body and beak varied from less than 110 µm to more than 240 µm, and in the same study, isolates of *A. alternata* gave the measurements of less than 37 µm to more than 69 µm (Rotem, 1966).

Based on morphological, physiological, and pathogenic variability of *A. solani*, eight races of the pathogen have been designated that differed in pathogenicity (Henning and Alexander 1959). Other researchers have differentiated races based on mycological criteria rather than pathological (Bonde, 1929). Those mycological criteria included various isolates, spore dimensions, sporulation capacity, and virulence. But the criteria of spore size found out to be misleading, as it varied between different cultures of the same race and among cultures of

different races. As previous studies indicated, differences among isolates of *A. solani* at morphological, physiological and pathogenic levels, it was important to determine variations at biochemical levels (Bonde, 1929; Henning and Alexander, 1969). Differences existing at the biochemical level were characterized using isozyme analysis (Petrunak and Christ 1992). Fifty four isolates of *A. solani* and 96 isolates of *A. alternata* were screened from various Solanaceous hosts and geographical locations using starch gel electrophoresis. Twenty three and 12 electrophoretic types were found for *A. solani* and *A. alternata*, respectively, possibly due to multi-host and quasi-saprophytic nature of the latter, and no significant correlation was found between isozyme phenotype and host or location of the isolates (Petrunak and Christ, 1992).

The similar characteristics among the small spore *Alternarias* make it difficult for accurate identification of Alternaria leaf spot diseases. Three small spore *Alternaria* spp. that are often confused with *A. alternata* based on morphology are *A. tenuissima*, *A. arborescens* (formerly *A. alternata* f. sp. *lycopersici*), and *A. infectoria*. (Taralova et al., 2011). Disputes of nomenclature within *A. alternata* make it difficult and confusing in pathogen identification. *A. alternata* isolates that are pathogenic on specific hosts, were designated into species epithet, such as *A. citri* and *A. mali* (Tymon, 2015). These species names are currently obsolete and they are classified as forma specialies of *A. alternata* (Rotem, 1994)

Genetic analyses of plant pathogen populations are critical in understanding epidemiology, host-pathogen coevolution, and management methods (Aradhya et al., 2001; Morris et al., 2000). Random amplified polymorphic DNA-PCR (RAPD-PCR) analysis was used to measure the genetic variation among isolates of the two pathogens (Weir et al., 1998). Extensive genetic diversity and significantly large genetic distances were discovered among the isolates of *Alternaria* spp., which might be an indication of pathogenic specialization of the

species (Weir et al., 1998). Virulence assays, vegetative compatibility (VC), and random amplified microsatellite (RAMS) techniques were used to determine genetic diversity among isolates of *A. solani* from various potato-growing regions in South Africa (Van der Waals et al., 2004). They found low virulence levels for the largest part of the population, but discovered high levels of diversity among isolates in VC tests and RAMS.

Biology of *Alternaria solani* and *Alternaria alternata*

Formation of conidia

Several *Alternaria* spp., such as *A. solani*, sporulate only after external induction, and in such cases formation of conidiophores and conidia can be examined independently of each other (Rotem, 1994). In contrast, *A. alternata* sporulates easily and usually do not require a trigger. *Alternaria* spp., as diurnal sporulators, are well adapted to the daily fluctuations in temperature and light (Leach, 1967). Diurnal sporulators use a mechanism of photosporogenesis with two distinctive phases. The first, or inductive, phase leads to the formation of conidiophores; the second, or terminal, phase results in the formation of conidia (Leach, 1967). The temperature and light requirements for the two phases are also distinct (Leach, 1967). The inductive phase is stimulated by near-ultraviolet (NUV) wavelengths in the range of 310 to 400 nm and relatively higher temperatures, while the terminal phase occurs in the dark and at lower temperatures, as it is strongly inhibited by NUV and blue light (Leach, 1967). When exposed to blue light and relatively high temperatures, conidiophores of *A. solani* arise from bud cells and form undifferentiated hyphal cells. When environment conditions change from light to darkness, the conidiophore produces a bud that turns into a conidium (Rotem, 1994). *A. alternata* sporulate without induction by forming conidiophores in darkness, so its sporulation cycle can be divided into three phases instead of two phases (Rotem, 1994). In the initial phase conidiophores are produced, conidiophores are triggered to form conidia in the induction phase, and finally, in the

terminal phase conidia are produced (Rotem, 1994). Although conidiophores can be formed in the presence of light or darkness, a brief exposure to NUV light is required to induce conidiophores to produce conidia.

The inhibition of the terminal phase of sporulation by light is a temperature-dependent process (Aragaki, 1961). In most cases, light inhibits sporulation when the temperature is relatively high. For instance, light inhibits the sporulation of *A. solani* when the temperature is between 26 and 31 $^{\circ}$C, but not at lower temperature of 20 $^{\circ}$C (Aragaki, 1961). In the *in vivo* sporulation of *A. solani* on potato, the inhibitory effect of light deceased with a drop in the incubation temperature from 25 to 15 $^{\circ}$C and with a decrease of the intensity of the light from 120 to 15.5 µE (Bashi and Rotem, 1975b). In contrast, illuminated cultures of *A. alternata* have been discovered to sporulate even at 31 $^{\circ}$C (Aragaki, 1964).

Optimum temperatures vary greatly for *in vitro* sporulation of the two *Alternaria* spp. An optimum temperature of 27 $^{\circ}$C has been reported for *A. alternata* (Pearson and Hall, 1975). It varies for *A. solani*, initial research (Rands, 1917) reported an optimum temperature of 26-28 $^{\circ}$C for sporulation, while 20 $^{\circ}$C was reported 27 years later (McCallan and Chan, 1944). Exposure to light affects optimum sporulation temperatures, as another researcher found optimum temperatures of 25 $^{\circ}$C for sporulation, of *A. solani* in light and 20 $^{\circ}$C with 16 h of light plus 8 h of darkness (Douglas, 1972). Temperature has similar effects on the production of conidiophores and conidia *in vivo* as it does *in vitro*. Conidiophores of *A. solani* on potato leaves develop in a wider range (5-35 $^{\circ}$C) with an optimum temperature of 22.5 $^{\circ}$C for sporulation (Rotem, 1994).

Apart from light and temperature conditions, other factors play a significant role in sporulation of *A. solani* and *A. alternata*. Sporulation is an aerobic process that requires oxygen and is inhibited by CO_2. But, *A. solani* is able to sporulate in 12 h in atmospheres nearly deplete

of oxygen that approach zero. *A. alternata* failed to sporulate when oxygen tension fell below 1% (Lukens and Horsfall, 1973). Conidial formation is highly sensitive to azide, dinitrophenol, and thiol reagents, but not to cyanide or fluoride. It was determined that conidial formation involves an iron-flavin terminal system with an affinity for oxygen with oxidative phosphorylation in the transformation of ADP to ATP (Lukens and Horsfall, 1973).

Sporulation of biotrophic pathogens is facilitated by a supply of photosynthates and survives on living tissue. But, the necrotrophic *Alternaria* spp. sporulate best on necrotic leaves and produce the maximum number of spores (Bashi and Rotem, 1975b). The inhibitory effect of photosynthates on sporulation of *A. solani* on potato was demonstrated by Israeli researchers (Cohen and Rotem, 1970). They explained that the inhibitory effect of photosynthates may be associated with the presence of sugars, which also reduce sporulation *in vitro* on sugar-rich media. Glucose enhances the production of conidiophores of *A. solani in vitro*, but inhibits the production of conidia (Waggoner and Horsfall, 1969). In the *in vivo* studies involving potato, glucose increased sporulation when applied to leaves during the formation of conidiophores, but inhibited spore formation on the already produced conidiophores. The association between starvation and sporulation can be ascribed to the effect of nonspecific stress (Cochrane, 1958). This association was supported 11 years later where the researchers suggested that nonspecific stress on non-germinated spores of *A. solani* produces secondary spores (Rotem and Bashi, 1969).

Due to the inhibiting effect of light on the terminal phase, sporulation in the field occurs mainly at night. Although most of the plant pathogens can produce spores within the wetting period of one single night, a majority of *Alternaria* spp. require a longer wetting period than one night (Rotem, 1994). However, some of the *Alternaria* spp. do not sporulate during long,

uninterrupted wetting, because they need induction by light or dryness (Rotem, 1994). The induction takes place between the first wetting period (the first night) where conidiophores are produced and the second wetting period (the second night) which is needed for the production of spores (Rotem, 1994). The two-night cycle of sporulation has been observed in *A. solani* on potato, which requires interrupted wetting periods (IWPs) to efficiently sporulate. The conidiophores during the two wetting periods tend to be in a state of minimal metabolic activity. The dry conidiophores of *A. solani* are capable of having "memorized" induction by light, producing spores after being stored in darkness for almost three weeks (Bashi and Rotem, 1976).

In some *Alternaria* pathogens, sporulation in the field is enhanced by rain. Although heavy dew allowed sporulation of *A. solani* on potato in Wisconsin, the most sporulation occurred when dew was supplemented with rain (Rands, 1917). The role of rain was demonstrated by displaying the partial inhibition of production of spores on existing conidiophores in filter paper cultures of *A. solani* (Waggoner and Horsfall, 1969). In most potato production areas, morning dews, rain showers, and high relative humidity create favorable conditions for the induction of sporulation (Holm, 2000).

Infection Process

The infectious period is the number of days during which spores are produced on previously infected tissue (Zadoks and Schein, 1979). The length of the infectious timeframe affects the number of spores produced and is a factor in plant pathogens' survival (Cohen and Rotem, 1987). The infectious period for *Alternaria* spp. is long, because infection mechanism starts in living leaves and continues after the leaves die. Temperature and humidity are vital environmental conditions that affect the infectious period. *A. solani* in potato sporulated for 12 and 21 weeks in leaves under dry conditions at 29 and 20 $^{\circ}$C, respectively, demonstrating the effect of temperature (Bashi and Rotem, 1975b). The infectious period of *A. alternata* on cotton

leaves lasted for 40 days and in the stems for 68 days (Rotem, 1994). The lesser infectious period for leaves is compensated with a higher number of conidia produced (5×10^4 spores per square centimeter lesion surface), and stem lesions produced (3.8×10^4). A similar trend was observed in *A. solani* on tomato and is apparently typical for *Alternaria* spp. that infect both leaves and stems (Rotem, 1994). The longer infectious periods can be an element to the senescence and sloughing of leaves with advanced infection (Wharam, 2002).

Germination

Most of the time conidia of the *Alternaria* spp. germinate without the addition of extrinsic nutrients, but in some specific conditions a supply of nutrients may improve germination (Rotem, 1994). The presence of free moisture and high relative humidity (RH) improves the germination of *Alternaria* spp. The germination of *A. solani* conidia exposed to ≥ 92% RH was associated with microscopic condensation of water (Stevenson and Pennypacker, 1988). In the same study under controlled temperature and moisture conditions, spores germinated more frequently in darkness when ambient temperature was near 25 °C and at ≥ 96% RH. The germination process of *A. alternata* occurs over a much wider range of temperatures than the infection process. The minimum temperature for germination ranges from 5-10 °C and the maximum ranges from 35-40 °C (Malathrakis, 1983; Norse, 1973). The optimum temperature for germination is approximately 29 °C (Malathrakis, 1983). For *A. solani*, the optimum temperature for germination is approximately 25 °C and the maximum temperature is approximately 35 °C (Bashi and Rotem, 1974). The spore germination of *Alternaria* spp. can be altered by changing metabolic pathways. Antimycin A slows the germination of *Alternaria* spores and further slowing can be caused by salicyl hydroxamic acid (SHAM), which inhibits the alternative oxidase pathway of fungal respiration (Waggoner and Parlange, 1977).

Penetration

Under favorable environmental conditions, most *Alternaria* spp. germinate in approximately 1-3 h, but penetration of host tissue by germ tubes may take much longer (Rotem, 1994). Density of stomata and thickness of the cuticle are the characteristics of the leaf surface that affect penetration. Pathogens penetrate mainly through the stomata-rich lower surface rather than the stomata-poor upper surface (Rotem, 1994). *A. solani* penetrates the epidermis or enters through stomata without signs of killing the invaded cells (Rotem, 1994). *A. alternata* penetrates potato leaves directly or via stomata, followed by extended developments causing a necrosis in the epidermal cells (Droby et al., 1984a). The pathogen penetrates bean leaves in a similar manner by forming an appresorium, which is a large structure assisting the penetration in terms of force (Saad and Hagedorn, 1969). In contrast, the germinating hyphae of *A. alternata* tend to spread over the intact leaf surface of the young, resistant tobacco plants without penetrating it (Rotem, 1994).

Wounded potato tubers may be penetrated at the harvest and are susceptible to infection by *A. solani*. Non-wounded tubers are never infected, whether covered with water or not (Venette and Harrison, 1973). Interactions among wetting period, temperature, and inoculum dose on the level of infection on potato leaves by the early blight pathogen were investigated by Waggoner and Horsfall (1969). Under favorable conditions (wetting period of 48 h and inoculum dose of 1×10^4 spores per cm^2), the minimum temperature for infection could be decreased from 20 to 10 $^{\circ}$C. These results confirm that *A. solani* is able to germinate as well as penetrate even during IWPs (Bashi and Rotem, 1974).

Enzymes

Enzymatic activity is essentially the most important method by which a plant pathogen penetrates a host and colonizes. But, effects of enzymes *in vitro* may differ from effects *in vivo*. For instance, cellulase demonstrated high activity in culture filtrates of *A. solani* and *A. alternata,* but during pathogenesis in tomato fruits it was less important than other enzymes (Mehta et al., 1974). Polygalacturonase (PG), polymethylgalacturonase (PMG), and cellulase (C_x) are highly active during the infection process of *A. solani* (Chaurasia et al., 2014). *A. alternata* produces pectin methyl lyase and C_x in causing early blight in tomato (Mehta et al., 1974).

Toxins

The majority of *Alternaria* spp. produce nonspecific toxins that are less damaging to plants than host-specific toxins (HST) and is not required for infection. *A. solani* produces nonspecific toxins including zinniol, alternaric acid, and several variations of altersolanol and macrosporin (Brian et al., 1952; Cotty and Misaghi, 1984). Zinniol production tends to be a common characteristic of large-spored, long-beaked *Alternaria* spp., which might be an indication of pathogenesis (Cotty and Misaghi, 1984). Alternaric acid, when introduced to tomato plants, caused chlorosis and necrosis and also caused damage to non-hosts such as cabbage, spinach, tobacco, bean, and sweet clover (Pound and Stahmann, 1951). Alternaric acid was detected in spore germination fluids and the substance alters morphological and physiological characteristics of plasma membranes near plasmodesmata (Langsdorf et al., 1990). These alterations cause permeability changes that contribute to leakage of electrolytes. There is a correlation between the quantities of alternaric acid and the amount of mycelium formed; no correlation was found between virulence of different strains of the pathogen and their ability to produce the acid (Brian et al., 1952).

A. alternata has seven pathogenic variants (pathotypes), which produce various HSTs and cause disease on different species (Takaoka et al., 2014). AM-toxin of the apple pathotype, AK-toxin of the Japanese pear pathotype, ACR-toxin of the rough lemon pathotype, AF-toxin of the strawberry pathotype, ACT-toxin of the tangerine pathotype, AT-toxin of the tobacco pathotype, and AAL-toxin of the tomato pathotype are the pathotypes of *A. alternata* (Tsuge et al., 2013). A host-specific toxin produced by the pathogen on potato has yet to be identified. HST plays a role in transitioning wild *A. alternata* from nonspecific and nonpathogenic to a pathogenic and host-specific type (Rotem, 1994). *A. alternata* also produces a variety of nonspecific toxins in tomato and pepper, with tenuazonic acid being the main mycotoxin along with alternariol, alternariol monomethyl ether, and altenuene (Stinson et al., 1981).

Symptomatology

Symptoms of early blight caused by *A. solani*, are initially observed in lower aging (senescing) leaves, the lesions first appear as irregular to circular, dark brown to black, small (1-2 mm in diameter) spots (Stevenson, 1993). The small spots coalesce, along with development of alternating series of light tan and dark concentric rings and depressed necrotic tissue, giving the lesions a target spot or bull's eye appearance (Secor and Gudmestad, 1999). Lesions become angular in shape when expansion is limited by leaf veins and are often surrounded by a narrow chlorotic halo (Draper et al., 1994). Eventually, heavily infected leaves turn yellow and senesce, but remain attached to the plant. As the disease begins to spread, small lesions appear on the younger leaves, along with dark, oblong lesions on stems and petioles (Franc and Christ, 2001). The symptoms are more severe on vines dying from natural aging and stressed by other diseases.

Potato tubers become infected during harvest, but symptoms do not become evident until months of storage. Tuber lesions are about six mm deep, dark, irregular shaped, sunken, and with a raised violet border (Secor and Gudmestad, 1999). The flesh underneath the lesion is dry and

often brown to black in color. Severely decayed tubers usually appear water soaked and yellow to greenish-yellow in color (Franc and Christ, 2001). Tubers could become shriveled in storage at high temperatures, due to enlargement of lesions (Venette and Harrison, 1973).

A. alternata is often found on aging lesions previously created by *A. solani*, due to its saprophytic nature (Rotem, 1994). However, it is the cause of brown spot disease of potato and causes black pit disease on tubers (Droby et al., 1984a, b). The pathogen was isolated from characteristic spots on potato leaves and Koch's postulates was completed, verifying that *A. alternata* is the causal organism. Brown spot lesions are similar to early blight, but tend to be smaller and darker in color (Gevens, 2012). Although early blight lesions initially occur in the older, lower canopy, brown spot typically appears first in the mid-canopy. However, the major comparison between the two diseases is that brown spot lesions never develop concentric rings, the major symptom of early blight (Kirk and Wharton, 2012). The brown spot lesions coalesce across large veins until whole leaves turn brown and remain attached to the plant. The tuber symptoms appear as small black holes, thus generally referred as black pit.

Epidemiology

<u>Overseasoning</u>

A. solani can survive in crop debris, soil, in infected tubers and in overwintering debris of susceptible Solanaceous crops and weeds, such as hairy nightshade (*Solanum sarrachoides*) (Rotem, 1968). Survival in debris is affected by meteorological, edaphic, and biotic conditions specific to each location, in most of the potato producing areas the pathogen overwinters more successfully as mycelium rather than conidia (Rotem, 1994). The conidia in infected potato leaves are able to survive freezing temperatures on the soil surface or buried to depths of 5 to 20 cm, and act as source of primary infection of succeeding crops (Rands, 1917). The extended viability may be due to dark pigmentation of hyphae, which increases their resistance to lysis

(Lockwood, 1960). Mycelium of *A. solani* survived a day at 88 °C and for over 10 years at 5 °C under controlled conditions of darkness and dryness (Rotem, 1968). Furthermore, the UV portion of solar radiation affects the survival of the pathogen than adverse temperatures in darkness, as survival in sunlight is about 30 times shorter than in darkness (Rotem, 1968). In the field, the fungus is protected from UV wavelengths by the upper leaves of the plant (Rotem, 1994).

Fruiting bodies of chlamydospores are associated with the mycelium and conidia of *A. solani*, which were discovered during examining early blight symptoms of tomato (Basu, 1971). Thick-walled, dark brown, round chlamydospores can range in diameter from 8 to 15 µm, and occur in chains, clusters, or singly (Basu, 1971). These propagules also enable *A. solani* to survive and overwinter in soil, with or without host tissues, through soil and air temperatures ranging from -3.3 to 21.1 °C and -31.1 to 27.7 °C, respectively, for seven months or more (Basu, 1971). Chlamydospores can cause primary infection to the next crop, but they are not produced by the pathogen frequently (Patterson, 1991).

<u>Dispersal</u>

The conidia of *A. solani* and *A. alternata* are dispersed mainly by wind along with splashing rain, and overhead irrigation. The larger conidial bodies of *Alternaria* spp. enable them to float in the air (Gregory, 1973). Conidia dispersal is high during periods when the weather is conductive to spore production. Wind velocity, along with dryness, are the two most important factors affecting conidial release from diseased tissues (Rotem, 1994). Dispersal is high during drier, warmer, and windier conditions, while dispersal is minimal on humid and windless nights. Storm conditions can also increase the degree of dispersal, as strongly attached younger conidia become detached (Rotem 1994). Typically, not all the conidia produced in one night are dispersed the following day; some of the conidia are retained on the foliage to create a reserve that accumulates in periods of low winds (Rotem, 1964).

Disease cycle

Alternaria spp. overwinters mainly as mycelium in crop debris, soil, infected tubers, or other Solanaceous hosts for five to eight months. The primary inoculum of conidia are produced in the spring (Van der Waals et al., 2003). Conidial dispersal occurs during dry, windy conditions and are readily moved within and between fields. Conidia landing first on fully expanded leaves near the soil germinate in the presence of free moisture from rain, irrigation, dew or high RH (around 95%) and favorable warm temperatures (20 to 30 °C) (Rotem, 1994). The pathogen may penetrate the epidermis directly, through stomata or wounds caused by sandstorms, mechanical injury or insect feeding (Rotem 1994). Lesions begin to initiate two to three days later, and subsequent formation of conidia and lesions occur within the growing season. The conidia produced by the primary infection is responsible for the secondary spread of the fungus to neighboring leaves and adjacent plants. Secondary infection is a typical scenario of a polycyclic disease, which progressively increases the rate of the foliar disease. Early blight is prevalent on senescing plant tissue and plants stressed from injury, low nitrogen, and pest pressure. Tubers can be infected during harvest due to mechanical injury (Venette and Harrison, 1973).

Disease epidemics may be induced by different weather factors in different locations. For instance, in Wisconsin, sporulation was enhanced by rain rather dew (Rands, 1917), and in Israel by dew rather than rain (Rotem, 1964). The rate of early blight development on potato is related to the susceptibility of cultivars, duration of leaf wetness, and temperature (Holley et al., 1985). They suggested that cultivar resistance and duration of leaf wetness are the important factors, rather than temperature in the predicting apparent infection rate. Major disease outbreaks do not occur until late in the growing season, due to relative resistance of young to intermediate-aged potato plants (Rotem, 1994)

Disease Management

The diseases of early blight and brown spot are potential threats where potatoes are grown under irrigation and during times of heavy dew (Rotem, 1994). Primary damage is attributed to premature defoliation of the potato plants, resulting in tuber yield reduction. Yield losses vary by location, cropping season, cultivar, and the stage of potato maturity (Olanya et al., 2009). Yield reductions of 5 to 40% were reported in Israel (Rotem and Feldman, 1965) and 20 to 30% in the USA (Shtienberg et al., 1990). The pathogens may also cause dry rot of tubers, further reducing both the quantity and quality of marketable tubers (Nnodu et al., 1982a). A combination of cultural and chemical measures are used to slow the development of disease and reduce the impact on the high value crop.

Cultural practices

Although cultural practices are not sufficient to suppress the diseases caused by *A. solani* and *A. alternata*, they can reduce the impact on the potato crop. Selecting fields for potato production is an important factor in minimizing disease potential. Fields with good drainage and fertility minimize plant stress and, therefore, reduce the susceptibility to the disease. Overhead irrigation enhances development of early blight by increasing the leaf wetness period, so it should not be applied at night, as this may increase dispersal of, and infection by, the pathogen (Van der Waals et al., 2003). Excessive water may also leach nitrogen from the soil and cause plant stress. It is also important to promote plant health and growth through balanced fertilization (Stevenson, 1993). Balanced nitrogen fertilizer application is essential to obtain mature tubers with proper skin-set at harvest time, which may decrease wounding during harvest and handling (McKenzie, 1981). Elimination of infected plant debris and weed hosts also help to reduce the inoculum level, because *A. solani* and *A. alternata* survive in plant debris in the field from one

growing season to the next. Crop rotation on a 3- to 5-yr schedule using non-host forage crops and grains reduces the amount of initial inoculum for disease initiation (Madden et al., 1978).

Another important cultural control method is minimizing tuber injury during harvest to prevent tuber rot in storage (Venette and Harrison, 1973). Farmers should harvest fully mature tubers, and avoid bruising or any other mechanical damage during harvesting and handling. Suberization and wound periderm development heal wounded surfaces of potato tubers (Nnodu et al., 1982a). These mechanisms protect tubers against invasion by pathogens and excessive evaporation. The rapidity of the formation of these barriers is important, as pathogens can enter healthy tissues until healing is complete (Nnodu et al., 1982b). The storage environment should be managed to facilitate rate and extent of wound healing, thereby reducing infection of tubers. Wound healing processes are largely dependent on environmental factors, especially relative humidity and temperature. The wounded tuber surfaces take approximately two days to heal at high relative humidity and temperatures (Nnodu et al., 1982a). Early storage conditions of tubers at 15.6 $^{\circ}$C for three weeks produced fewer and smaller lesions than constant storage at 10 $^{\circ}$C and 4.4 $^{\circ}$C (Nnodu et al., 1982b). Providing a proper storage environment immediately after harvest is essential for wound healing of potato tubers (Venette and Harrison, 1973).

Host resistance

Planting cultivars that are less susceptible to early blight and brown spot may also reduce disease severity. Permanent resistance is not affected by the conditions of growth, but temporary resistance is largely determined by plant age, the rate of cultivar maturity, and yield (Rowell, 1953). Incubation period, lesion expansion rate, spore production, and receptivity of the tissue to infection were the components of resistance in three potato cultivars tested (Pelletier and Fry, 1989; 1990). Only the component of sporulation was found to be age-independent, reflecting the permanent resistance of the plants.

Computer simulation models have been used to determine the relative contribution of genotype resistance, age-related resistance, and fungicides in early blight suppression (Shtienberg et al., 1995). Although physiological cases of age-related resistance have yet to be determined, a theory correlating the concentration of sugar present in the plant tissue to the susceptibility to the pathogens was introduced (Horsfall and Dimond, 1957). They claimed that plant tissue that is low in sugar becomes resistant to biotrophic pathogens, with are associated with “high-sugar” diseases. However, plant tissue that is low in sugar becomes susceptible to necrotrophic pathogens such as *Alternaria* spp, which are associated with “low-sugar” diseases. Late in the season non-reducing sugars are directed towards to the formation of tubers, therefore, reducing the amount of sugar in the foliage (Shtienberg et al., 1995). Senescence is also associated with other biochemical processes such as decreased levels of the alkaloid solanine, which inhibits *A. solani in vitro* (Sinden et al., 1973).

Most potato breeding lines with field resistance to *A. solani* are low-yielding and late-maturing genotypes (Barksdale, 1971). But sources of genotype resistance to the pathogen in *S. tuberosum* are relatively rare and genotype resistance is not the only factor determining host response to early blight. Several potato cultivars were evaluated for disease resistance under field and greenhouse conditions and significant differences were observed among several cultivars for disease reaction against the pathogen (Christ, 1991). Over the three-year period, the late-maturing cultivars of Katahdin and Kennebec were more resistant to *A. solani* than the early-maturing cultivars of Norland and Superior, but not necessarily more resistant than the midseason-maturing cultivars Atlantic and Chieftain (Christ, 1991).

Breeding potato cultivars with early blight resistance is a major method to combat disease in cultivated potato. A large study of 934 potato clones from around the world identified few

cultivated potato genotypes with early blight resistance (Boiteux et al., 1995). Many breeding programs are using wild *Solanum* spp. germplasm as a source for resistance genes to develop populations with resistance to multiple diseases (Jansky and Rouse, 2003). An early blight resistant clone of the diploid wild species *S. raphanifolium* was crossed as a male to a haploid (2n=2x) of cultivated potato (Weber and Jansky, 2012). The progeny created by backcrossing to the wild species parent demonstrated significantly lower relative area under disease progress curve (RAUDPC) means than those from backcrossing to the cultivated parent. Both laboratory assays (Jansky et al., 2008) and field studies (Weber and Jansky, 2012) confirmed that the wild species *S. raphanifolium* exhibits a high level of early blight resistance. Although small lesions were observed in field studies, the researchers could not isolate *A. solani* from the lesions. This phenomenon explains that if the pathogen cannot grow and reproduce on the potato leaves, then the wild species contain a resistance factor (Weber and Jansky, 2012). Plants containing the resistance factor are protected, there is a decrease in inoculum production, and disease pressure on adjacent fields with susceptible plants is less. Breeders now have a target wild species for resistance breeding rather resistance based on late maturity. The application of fungicides on a moderately resistant cultivar on a 17-day schedule suppressed disease at levels similar to those achieved by spraying a susceptible cultivar on a 7-day schedule (Shitienberg and Fry, 1990). Potato cultivars that are less sensitive to early blight and brown spot may reduce fungicide applications required to manage the diseases (Christ, 1991).

Chemical control

Application of foliar fungicides starting 6-7 weeks after planting is the most common and effective practice adopted worldwide to control early blight and brown spot (Christ and Maczuga, 1989). Frequent application of protectant fungicides from early in the growing season until vinekill is essential, especially when potatoes are grown under sprinkler irrigation in

intensive production systems (Stevenson, 1994). Good coverage is also essential, as early blight and brown spot initiate in the lower canopy. Protectant fungicides recommended for late blight (*Phytophthora infestans*) control (e.g., chlorothalonil, mancozeb, metiram, and copper hydroxide) are also efficient against early blight and brown spot when applied at approximately 7 to 10 day intervals (Holm et al., 2003). Application of foliar fungicides is not needed for early blight management in plants at the vegetative stage when they are relatively resistant (Shtienberg et al., 1996). The application of fungicides should initiate only when host response to *Alternaria* shifts toward increased susceptibility. Therefore, the initial fungicide application should occur at the first sign of disease or immediately after bloom (Draper et al., 1994). Follow-up sprays should be determined according to the genotype and age-related resistance of the cultivar, and the efficacy of the fungicide (Shtienberg et al., 1996). Protectant fungicides should be applied initially at longer intervals and subsequently at short intervals as the crop ages. Early-season applications before secondary inoculum is produced often have minimal or no effect on the spread of the disease. The pathogens can be adequately controlled by relatively few fungicide applications if the initial application is properly timed using forecasting models, therefore, reducing costs (Douglas and Groskopp, 1974; Harrison et al., 1965).

Disease forecasting models have been developed to predict the onset of disease and specify when the initial fungicide application should be used. EPIDEM is rather a simulator to detail quantitative analysis of all the *A. solani* life cycle stages (Waggoner and Horsfall, 1969). A computerized forecasting system for *A. solani* on tomato (FAST) was developed in Pennsylvania to identify periods when environmental conditions are favorable for early blight development and to provide an efficient fungicide application schedule (Madden et al., 1978). Several forecasting models for early blight on potato were evaluated in Wisconsin, along with FAST,

which uses temperature, relative humidity, rainfall, and leaf-wetness to calculate severity values (Pscheidt and Stevenson, 1986). The same research group also focused on identifying the critical period for initiating the fungicide spray schedule based on physiological days (P-Days) of plants, which is a method of measuring useful heat for the growth of potato (Pscheidt and Stevenson, 1988). P-Days accumulate from time of emergence and incorporate three temperature thresholds that represent the minimum, optimum, and maximum temperatures for potato (7, 21, and 30 °C) and the diurnal fluctuation of air temperature (Pscheidt and Stevenson, 1986). In general, early-maturing cultivars should be sprayed at approximately 250 P-Days and spraying of late-maturing cultivars should begin at approximately 300 P-Days (Stevenson, 1993). A P-Day of 300 timing correlates with initial increase in early blight spore concentration and typically aligns with row closure (Gevens, 2012). A simple model was developed to predict time of secondary sporulation of *A. solani* based upon accumulated degree days (DD) above 7.2 °C from the date of planting (Franc et al., 1988). This model is useful to time the initial fungicide application to reduce the cost of disease management. Regular scouting of fields after plants reach 12 inches in height is suggested to identify early infections (Wharton and Kirk, 2007).

Chlorothalonil (Bravo®, Echo®, and Equus®) is a substituted benzene compound with broad-spectrum activity. It has a multi-site mode of action, which inhibits the formation of sulfur-containing enzymes (Sujkowski, 1995). The chemical class known as the ethylenebisdithiocarbamates (EBDC) include the fungicidal products mancozeb (Manzate®, Dithane®, and Penncozeb®) and metiram (Polyram®).This preventive chemical class also has broad-spectrum activity and a multi-site mode of action (Holm, 2000). The EBDCs break down to cyanide, which reacts with thiol compounds in the cell and interfere with sulfhydryl groups (Georgopolus, 1977). These protectant fungicides are generally effective in controlling early

blight, and have a minimal resistance risk due to multi-site modes of action. In contrast to these protectant fungicides, triphenyl tin hydroxide (TPTH) has a limited spectrum of fungistatic activity. It provides control of several diseases, including early and late blight, brown spot, scab, leaf blotch, powdery mildew, and others (Holm, 2000). TPTH fungicides (Super Tin® and Agri Tin®) destroy cell membranes, therefore inhibiting the respiration process (Georgopolus, 1977). This chemical group also has a multi-site mode of action similar to other preventive fungicides, making it difficult for fungi to develop resistance. However, application of protectant fungicides are insufficient at high inoculum pressure and conducive environmental conditions that enable distribution and development of the pathogen (Pasche and Gudmestad, 2008).

The quinone outside inhibiting (QoI) fungicides categorized into Group 11 by the Fungicide Resistance Action Committee (FRAC) were first introduced in 1999 on potato and provided excellent disease control (Pasche and Gudmestad, 2008; Stevenson and James, 1999). This fungicide class mainly consists of strobilurins that inhibit fungal respiration at mitochondrial complex III. QoI fungicides have a single site mode of action, interfering with the electron transport of the cytochrome bc_1 complex (Wong and Wilcox, 2000). The QoI fungicides represent an important class of agricultural pesticides for the control of a broad range of pathogens from all three major groups of fungi (Bartlett at al., 2002). The discovery of QoIs were initiated as a result of research on a family of natural derivatives of β-methoxyacrylic acid; the strobilurins, oudemansins, and myxothiazols (Wharam, 2002). The sales of QoIs totaled approximately $620 million in 1999, and represented over 10% of the global fungicide market (Bartlett at al., 2002).

Azoxystrobin (Quadris®, Satori®, and Equation®) is a broad-spectrum QoI fungicide with protectant, translaminar, and systemic (xylem only) properties to control early blight, brown

spot, late blight, and black dot (*Colletotrichum coccodes*) (Holm, 2000; Wharam, 2002). The Environmental Protection Agency (EPA) granted an emergency use (Section 18) label for azoxystrobin during the later stage of the 1998 growing season for North Dakota, Minnesota, Nebraska, and Wisconsin (Pasche et al., 2004). Full registration (Section 3) was granted for use on potato throughout the United States in 1999, and trifloxystrobin (Gem®) and pyraclostrobin (Headline®) were registered in 2001 and 2002, respectively. Non-strobilurin QoI chemistries of famoxadone (Tanos®) and fenamidone (Reason®) were registered in 2003 and 2004, respectively, and have modes of action similar to those of azoxystrobin (Pasche et al., 2005).

QoIs are particularly potent spore germination inhibitors, inhibit mycelial growth, as well as containing antisporulant activity. Due to its efficacy on inhibiting spore germination, azoxystrobin is most effective when used prior to infection, or during the early stages of disease development (Wharam, 2002). Soon after registration, producers in the midwest frequently performed four to six applications in a single growing season (Pasche and Gudmestad, 2008). Initially, excellent disease control was provided by this new chemistry. During 2001 to 2003, approximately 80% of the total potato acreage was sprayed with QoI fungicides with an average of three applications per year (Rosenzweig et al., 2008b). The reduced efficacy of azoxystrobin and pyraclostrobin to levels of disease control provided by chlorothalonil and mancozeb made them less attractive for disease control due to them being expensive compared to the protectant fungicides (Pasche and Gudmestad, 2008). The brown spot pathogen is inherently more resistant to QoIs and has not been well controlled by this fungicide chemistry (Fairchild et al., 2013).

The succinate dehydrogenase inhibitor (SDHI), systemic fungicide, boscalid (Endura®) was registered for the use on potato in 2005 and became a reliable alternative to QoI fungicides (Pasche and Gudmestad, 2008; Pasche et al., 2005). The target site of SDHI (FRAC Group 7)

fungicides, previously known as carboxamides, is the cytochrome b_{560} subunit of mitochondrial complex II, at either succinate ubiquinone reductase or succinate dehydrogenase (Sdh) in the respiratory chain of fungi (Cecchini, 2003; Kuhn, 1984). The broad spectrum fungicide does not inhibit the succinate reductase activity of the complex II, but the quinine reduction activity (Avenot and Michailides, 2007). So, unlike most other fungicides, boscalid has an unusual fungicidal profile. The enzyme complex is a functional part of the tricarboxylic acid cycle and the mitochondrial electron transport chain (Yin et al., 2011). It also catalyzes both the oxidation of fumarate and the reduction of quinine (Avenot and Michailides, 2007).The Sdh complex in fungi is composed of four subunits. SdhA is a flavoprotein (Fp), whereas SdhB is an iron-sulfur protein (Ip) containing three different iron-sulfur clusters (S1, S2, and S3), and two hydrophobic membrane-spanning subunits of SdhC and SdhD (Avenot and Michailides, 2010). The Fp and Ip subunits form the soluble part of the complex and carry the Sdh activity. The SdhC and SdhD subunits anchor Fp and Ip to the membrane and have quinone reducing activity (Ito el al., 2004).

SDHI fungicides through inhibition of mitochondrial complex II, interfere with spore germination, mycelial growth, germ tube elongation, and sporulation of various plant pathogens such as *Botrytis cinerea*, *Monilinia fructicola*, *Corynespora cassiicola* (Amiri et al., 2010; Miyamoto et al., 2010; Myresiotis et al., 2007). In research spray programs, at least one application of boscalid, significantly increased disease control and overall yield (Franc and Stump, 2008; Stevenson and James, 2007). Boscalid, a premium fungicide, is a pyridine-carboxamide SDHI that has been in use for almost ten years. Within three years of use, resistance to boscalid developed, the first case was reported in Idaho (Wharton et al., 2012).The EPA has registered several new fungicides containing SDHI active ingredients; penthiopyrad (Vertisan® and Fontelis®), fluopyram (Luna® and Luna Tranquility®) and fluxapyroxad (Xemium® and

Priaxor®). Penthiopyrad and fluxapyroxad are pyrazole-carboxamides, while fluopyram is a pyridinyl-ethyl-benzamide (FRAC, 2015). The differences in SDHI sensitivity to the three fungicides evaluated in laboratory trials are probably a result of differences in chemistries (Fairchild et al., 2013). Boscalid is a densely contorted, non-fluorinated compound, whereas fluopyram and penthiopyrad are fluorinated, long, linear compounds (Fraaije et al., 2012). This may help the binding sites of the latter fungicides to be more efficient in inhibiting complex II and makes them more competitive with ubiquinone for the ubiquinone pocket compared to its boscalid counterpart (Fairchild et al., 2013).

Penthiopyrad is a novel fungicide that shows activity against Basidiomycete pathogens *Rhizoctonia*, as well as Ascomycete pathogens of *B. cinerea* and *Venturia inaequalis* (Yanase et al., 2007). It is a unique fungicide chemistry containing both a pyrazole and thiophene ring. The main targets of penthiopyrad are spore germination and sporulation, but it also inhibits mycelial growth. Fluopyram was first registered for use on potato in 2012 and is highly anticipated for use in the regions where high levels of boscalid resistance has been detected. Fluopyram may also be more effective at inhibiting *A. solani* growth *in vitro* due to multiple molecular configurations of the compound and its competitiveness increases for the active site of the SDH complex and allow it to interact with more amino acid residues (Musson and Young, 2012). It is biologically active against all stages of growth, from spore germination to sporulation, and is active against a broad range of pathogens in Ascomycetes (Veloukas and Karaoglanidis, 2012). Fluxapyroxad has both preventive and curative properties in inhibiting spore germination, germ tube growth, mycelial growth, and appresoria formation of major phytopathogenic fungi. Priaxor® is highly effective in controlling early blight in both tomato and potato, as well as powdery mildew

(*Leveillula taurica*) and brown spot in tomato, and black dot in potato, in the rate range of 146 – 300 g ai/ha (Strathman et al., 2011).

Demethylation inhibitors (DMIs) are one of four classes of sterol biosynthesis inhibitors (FRAC Group 3), first introduced in the 1970s, they have a broad spectrum action on a number of fungal pathogens from the Ascomycetes and Basidiomycetes (Thomas et al., 2012). DMIs have gained popularity because of their protective and curative properties, and low levels of phytotoxicity (Dahmen and Staub, 1992). Difenoconazole (Inspire® and Revus Top®) is a translaminar fungicide with durable preventive activity during penetration and haustoria formation of fungal plant pathogens causing various leaf spot diseases, powdery mildews, rust and scab of annual and perennial crops (Bouwman et al., 2011). Metconazole (Quash®), another DMI fungicide, has excellent activity on various smut and rust diseases, root rots, and powdery mildews (Friskop et al., 2015). Their mode of action is the inhibition of the C14 α-demethylation of 24-methylenedihydrolanosterol, a precursor of ergosterol in fungi (Brent and Holloman, 2007a). Inhibition of sterol biosynthesis in cell membranes of fungi causes disruption of membrane function, leakage of cytoplasmic contents, and hyphal inhibition. Difenoconazole and metconazole were first registered for use on potato in 2011. They have been key rotational fungicides in growers' spray programs to control the pervasive potato diseases.

Pyrimethanil (Scala® and Luna Tranquility®), an anilino-pyrimidine (FRAC Group 9) fungicide targeting methionine biosynthesis, was registered on potato for early blight disease control in 2005 (N. C. Gudmestad, *personal communication*). The APs also inhibit the secretion of hydrolytic enzymes, affecting the infection process of pathogens (Heye et al., 1994; Masner et al., 1994). Pyrimethanil and other AP fungicides have largely been used as broad spectrum foliar fungicides to control several pathogens including *B. cinerea* (Zhao et al., 2010), and *V.*

inaequalis (Köller et al., 2005) in vegetables, fruits, and cereals. Pyrimethanil has the potential to be very beneficial to fungicide application programs in the potato industry as a substitute for resistant chemistries such as QoI fungicides. It should be used in rotation with other fungicides to avoid resistance in areas of high disease pressure such as the midwest.

Fungicide Resistance

Fungicides remain critical for controlling plant diseases caused by fungi, which are estimated to cause yield reductions of approximately 20% in the major crops worldwide (Gullino et al., 2000). They are necessary for maintaining healthy, reliable, and high-quality agricultural products. The initial fungicides introduced prior to 1970 were multi-site inhibitor protectant fungicides. Preventive fungicides such as mancozeb, thiram, or maneb were widely used for many years, but no resistant strains of fungi have been observed. Since the introduction of single-site fungicides, fungicide resistance in phytopathogenic fungi has become a major obstacle in crop protection. The occurrence of fungicide resistance first appeared following the registration and continuous use of the systemic fungicide benomyl in the early 1970's (Damicone, 2009).

Fungicide resistance is a stable, heritable adoption by the fungus to a fungicide, resulting in a reduction of sensitivity by the fungus to the fungicide (Ma and Michailides, 2005). Resistance occurs when populations of a target pathogen once sensitive, are no longer sufficiently controlled. The speed and frequency of development of fungal resistance towards fungicides depends on factors such as mode of action of active ingredients, rate and frequency of fungicide use, population dynamics of the fungus, the propagation rate, and fitness cost of an acquired resistance (Klix et al., 2007). Fungicide-resistant fungal biotypes may occur naturally and may be distributed randomly throughout the population. Resistance is more likely to occur when one specific fungicide or chemicals with the same mode of action are used continuously.

Since sensitive biotypes are controlled, more resistant biotypes may become predominant in the pathogen populations under selection pressure of fungicide use over time (Ma and Michailides, 2005). The loss of efficacy builds up though the survival of initially rare mutants during exposure to fungicides (Brent and Holloman, 2007a). Pathogens that are polycyclic with short generation periods develop resistance faster than the others. The development of resistance can be discrete, resulting from a single gene mutation, or gradual, which is considered to be polygenic.

Many fungicides developed and registered after the late 1960s are systemic, with a site-specific mode of action. It means they are only able to inactive a key enzyme, or act on one point in one metabolic pathway in a pathogen (McGrath, 2001). When a rapid shift towards resistance occurs from a mutation of single major gene, it is considered a development of qualitative resistance (Damicone, 2009). When multiple genes are involved, the shift toward resistance develops slowly, resulting in quantitative resistance under the selection pressure of fungicide use. Mutations related to fungicide resistance tend to display negative pleiotropic effects, known as a fitness penalty, which become evident in the absence of fungicide selection pressure (Schoustra el at., 2005). The fitness of resistant isolates can be defined as the ability to survive and reproduce under the same environmental conditions as sensitive isolates (Karaoglanidis et al., 2001). The pathogenic fitness of resistant isolates affects the dynamics of competition between resistant and sensitive isolates and thereby, is the source of evolution of resistance in fungal populations (Karaoglanidis et al., 2011). The frequency of resistant populations may decline, if the fitness of resistant isolates are less than that of sensitive isolates in the absence of fungicide selection pressure. In contrast, absence of fitness penalty would lead to stable resistance in the

absence of fungicide selection pressure or increase of resistance frequency and loss of efficacy in the field in the presence fungicide selection pressure (Karaoglanidis et al., 2001).

Resistance to QoI fungicides

QoI fungicides were first discovered in 1992 and released for sale in 1996 (Bartlett et al., 2002). Since QoIs have a single site-specific mode of action, selection for resistant mutants of phytopathogenic fungi increases greatly. After two years of use, resistance was first discovered in cereals in isolates of *Erisphe graminis* DC f sp *tritici* in northern Germany (Bartlett et al., 2002). Since then, field resistance to QoI fungicides has been reported in over 40 phytopathogenic fungi (FRAC, 2012. Three amino acid substitutions that confer qualitative selection have been detected in the cytochrome b (*cytb*) gene in phytopathogenic fungi that govern resistance to quinone outside inhibitors (FRAC, 2006).

For most of the pathogens in which QoI resistance has been reported, the predominat single nucleotide polymorphism is glycine replaced by alanine at position 143 (G143A) (Heaney et al., 2000). This mutation has resulted in resistant phenotypes in *Plasmopara viticola* (Heaney et al., 2000), *Mycosphaerella fijiensis* (Chin et al., 2001), *V. inaequalis* (Steinfeld et al., 2002), *Pyricularia grisea* (Kim et al., 2003), *Alternaria* spp. (Ma et al., 2003), *Didymella bryoniae* (Stevenson et al., 2004), *Pyrenophora tritici-repentis* (Sierotzki et al., 2007), *B. cinerea* (Ishii et al., 2009), *Cercospora beticola* (Birla et al., 2012), and *Ascochyta rabiei* (Delgado et al., 2012). Fungal isolates carrying the G143A mutation express high (complete) resistance as resistance factors (RF= EC_{50} of the resistant strain / EC_{50} of sensitive strain) are in most cases greater than 100 (Chin et al., 2001). Severe or complete loss of disease management is observed in populations where G143A predominates and QoIs are used alone. This mutation also has been shown to provide cross-resistance among QoI fungicides in several phytopathogenic fungi (Ishii et al., 2001, Kim et al., 2003).

The second target-site mutation is the substitution of phenylalanine with leucine at position 129 (F129L) of the *cytb* gene. This mutation is observed in *P. grisea* (Kim et al., 2003), *A. solani* (Pasche et al., 2005), *P. teres* and *P. tritici-repentis* (Sierotzki et al., 2007), and *P. viticola* (FRAC, 2012). The third single point mutation results in a glycine to arginine change at position 137 (G137R). It was reported in two out of 250 isolates of *P. tritici-repentis* from 2005 (Sierotzki et al., 2007). The RF's caused by F129L and G137R mutations are under 50, thus express moderate (partial) resistance (FRAC, 2006). Although a severe loss in disease control is not observed, the mutations of F129L and G137R cause reduced disease control of target populations. In contrast to the G143A mutation, the F129L mutation has a differential effect on fungal sensitivity to QoI fungicides (Kim et al., 2003; Pasche et al., 2004, 2005). In addition to single nucleotide polymorphisms, fungi can also gain resistance to QoI fungicides by inducing an alternative respiratory pathway (Olaya and Köller, 1999). This mechanism is a response to overcome fungicidal effects of respiration inhibitors and is active in the presence of alternative oxidase. But alternative oxidase does not play a significant role in pathogenesis on QoI-treated plants in natural populations of pathogens controlled by these chemistries (Olaya and Köller, 1999).

A. alternata resistance to azoxystrobin has been reported in pistachio in California (Ma et al., 2003) and in citrus in Florida (Vega et al., 2012). Reduced-sensitivity of *A. solani* to QoI fungicides, especially azoxystrobin, was detected first in Nebraska in 2000 and in North Dakota and Minnesota in 2001 (Pasche et al., 2004, 2005) and spread across the region in the following years (Pasche and Gudmestad, 2008). QoI fungicides are inhibitors of spore germination, so a spore germination assay was developed to determine the sensitivity of *A. solani*. It is the first report of a fungal plant pathogen without a known sexual cycle building reduced-sensitivity

against QoI chemistries (Pasche et al., 2004). The survey conducted in these midwestern states showed an approximate 13-, 10-, and 2-fold decrease in sensitivity to azoxystrobin, pyraclostrobin, and trifloxystrobin, respectively. Cross-sensitivity assays showed that isolates with reduce-sensitivity to azoxystrobin also possess reduced-sensitivity to trifloxystrobin and pyraclostrobin, despite these fungicides not being registered for potato until 2001. In the *in vivo* trials, azoxystrobin and pyraclostrobin showed significantly less efficacy in controlling the disease caused by *A. solani* isolates with reduced-sensitivity to azoxystrobin. However, the amount of disease control provided by trifloxystrobin was not affected. In this survey, the term "reduced-sensitivity" was used instead of resistance, as resistance factors were determined to be significantly less in *A. solani* compared to other previously reported fungi (Pasche et al., 2004). A later study revealed the shift in sensitivity to famoxadone and fenamidone to between two- and three-fold, and initial *in vivo* studies also did not demonstrate any loss of early blight control caused by the F129L mutation. (Pasche et al., 2005).

During 2002 and 2003, a statewide survey of monitoring *A. solani* indicated a 20-fold shift in sensitivity to azoxystrobin and a wide distribution of the F129L mutation across the potato production regions of Wisconsin (Rosenzweig et al., 2008a). A survey conducted by an Idaho research group in 2009 and 2010 demonstrated 100% of *A. solani* isolates and 75% of *A. alternata* isolates were resistant to azoxystrobin (Fairchild et al., 2013). They used a spiral gradient dilution method, instead of a spore germination assay to determine *in vitro* fungicide sensitivity. Sensitivity to other QoI fungicides differed; 60%, 15%, 78%, and 86% of *A. solani* isolates were resistant to trifloxystrobin, pyraclostrobin, picoxystrobin and famoxadone, respectively, and similar results were reported for *A. alternata* (Fairchild et al., 2013). QoIs were registered for use on potato in 2007 in Germany, and two years later, reduced-sensitivity to

azoxystrobin, as well as to pyraclostrobin, were reported in the *in vitro* spore germination assays (Leiminger et al., 2013). The frequency of reduced-sensitive isolates increased over the years 2009 to 2011, and findings were correlated with the loss of disease control in the *in vivo* studies.

The reduced-sensitivity to QoI fungicides demonstrated by *A. solani* is due to the F129L mutation, this substitution is caused by one of the three nucleotide mutations (TTA, CTC, and TTG) (Rosenzweig et al., 2008a). Furthermore, due to diversity of the *cytb* gene, two different genotypes were found among *A. solani* isolates (Leiminger et al., 2013). The two genotypes differ in the presence (Type I) or absence (Type II) of an intron. By 2013, QoI resistance was widespread, as our research group found the F129L mutation in 99% of *A. solani* isolates recovered from early blight infected potato leaves from all major potato producing regions in the United States.

Resistance to SDHI fungicides

The SDHI group of fungicides, originally called carboxamides were among the first systemic fungicides released (Russell, 2005). Carboxin, a narrow-spectrum fungicide was introduced in 1966 as a systemic seed treatment for *Rhizoctonia* and other Basidiomycete pathogens of smuts and bunts (Sierotzki and Scalliet, 2013). The first truly broad-spectrum foliar SDHI fungicide was boscalid, launched in 2003. Initially, boscalid was an effective substitute for QoI fungicides, in controlling many phytopathogenic fungi, including early blight of potato (Pasche and Gudmestad, 2008). Fungicides in the SDHI group are considered to be at medium to high risk for development of resistance, because of their single-site mode of activity (FRAC, 2015).

The first report of carboxin resistance was reported in *Ustilagio maydis* in 1975 (Georgopoulos et al., 1975) and also in the wheat plant pathogen *Mycosphaerella graminicola*, that causes septoria leaf blotch (Skinner et al., 1998). Since then, the frequent use of SDHI

fungicides has caused resistance in various pathosystems such as *A. alternata* of pistachio, potato, and peach (Avenot and Michailides, 2007; Fairchild et al., 2013; Tymon and Johnson, 2014; Yang et al., 2015), *B. cinerea* of several crops (Bardas et al., 2010; Leroch et al, 2011; Yin et al., 2011*), C. cassicola* of cucumber (Miyamoto et al., 2010), *D. bryoniae* of cucurbits (Thomas et al., 2012), *M. fructicola* of peach (Amiri et al., 2010), *Podosphaera xanthii* of cucumber (Ishii et al., 2011), and *A. solani* of potato (Gudmestad et al., 2013; Tymon and Johnson, 2014; Wharton et al., 2012).

The mutations reported with SDHI resistance are in the subunits of SdhB, SdhC, and SdhD. In *U. maydis*, replacement of highly conserved histidine residue by either tyrosine or leucine at position 257 (H257Y/L) is correlated with carboxin resistance in the SdhB subunit (Keon et al., 1991). Similar resistance is reported in *M. graminicola*, with a replacement occurring at position 267 (H267Y/L) (Skinner et al., 1998). In the closely related species of *A. alternata*, single-point mutations in the SdhB subunit (H277Y/R) have been reported (Avenot and Michailides, 2010). Replacements of histidine by either tyrosine or arginine at position 277 conveys moderate or high level of resistance to boscalid and low, moderate, or high levels of resistance to penthiopyrad and fluxapyroxad (Avenot et al., 2014). Molecular characterization results show different sensitivities and cross-resistance patterns between structurally different SDHIs. In contrast to the other SDHI chemistries, fluopyram controlled all the SdhB mutants in *A. alternata* (Avenot et al., 2014)

Low to high levels of boscalid resistance is conveyed in the SdhB subunit in *B. cinerea* (H272Y/R) (Yin et al., 2011). Those mutants remain sensitive to fluopyram and fluxapyroxad, but the status of the shift of sensitivity to penthiopyrad has not been reported yet. A similar situation exists in *C. cassicola* (H278Y/R) (Miyamoto et al., 2010) and *D. bryoniae* (H277Y/R)

(Avenot et al., 2012). The less frequent mutations of histidine and proline replacements by leucine (H272L) and leucine or phenylalanine (P225L/F), respectively, seem to confer high levels of resistance to all four SDHI fungicides in *B. cinerea* (Amiri et al., 2014; Veloukas et al., 2011). A moderate level of resistance to boscalid in *B. cinerea* was found to be associated with the replacement of proline by threonine at position 225 (P225T) and of asparagine by isoleucine at position 230 (N230I) (De Miccolis Angelini et al., 2014).

Single point mutations in the SdhC subunit (H134R), replacement of histidine by arginine at position 134 and in the SdhD subunit (H133R), and asparate replacement by glutamic acid at position 123 (D123E), convey high level of boscalid resistance in *A. alternata* (Avenot et al., 2009). These mutants demonstrated variable patterns of cross-resistance among other SDHI fungicides (Avenot et al., 2014). No mutations in the SdhC subunit have been associated with boscalid resistance in *B. cinerea*; in the SdhD subunit, replacement of histidine by arginine at position 132 (H132R) conferring resistance to boscalid has been reported in a limited number of field isolates (Leroux et al., 2010). Replacements of serine by proline at position 73 (S73P) in the SdhC subunit, at position 89 (S89P) in the SdhD subunit and glycine by valine at position 109 (G109V) also in the SdhD subunit, convey moderate resistance to boscalid in *C. cassicola* (Miyamoto et al., 2010).

The first report of boscalid resistance in *A. solani* was reported in 2009 and 2010 in Idaho, with 15 and 62% of the isolates insensitive to the fungicide, as well as 56 % of *A. alternata* isolates (Fairchild et al., 2013; Wharton et al., 2012). Additionally, resistant isolates were prevalent in North Dakota, Minnesota, Nebraska, Texas, Colorado, Wisconsin, and Florida in 2010 and 2011 (Gudmestad et al., 2013). Almost 80% of *A. solani* isolates showed resistance to boscalid with two phenotypes, or levels of phenotype of resistance. Five and 75% of all

isolates of the population were moderately resistant (5 to 20 µg/ml), representing a 15- to 60-fold loss in sensitivity and highly resistant (>20 µg/ml), representing a >100-fold loss in sensitivity, respectively, to boscalid (Gudmestad et al., 2013). All of the isolates remained sensitive to fluopyram and also remained predominantly sensitive to penthiopyrad. The efficacy of boscalid was similar to the level of disease control provided by the QoI fungicides azoxystrobin and pyraclostrobin in managing *A. solani* isolates with the F129L mutation (Pasche et al., 2004; 2005). Therefore, the level of control boscalid provides would be similar to the level of control provided by protectant fungicides such as chlorothalonil and mancozeb (Pasche and Gudmestad, 2008). In this *in vivo* trial, fluxapyroxad failed to control the disease except at the highest concentration of the fungicides (Gudmestad et al., 2013). Thus, we can assume *A. solani* isolates possessing boscalid resistant are cross-resistant to fluxapyroxad.

A study conducted in Idaho in 2010, showed several boscalid resistant *A. solani* isolates to be cross-resistant to penthiopyrad, but none showed resistance to fluopyram (Fairchild et al., 2013). But isolates collected from the 2011 growing season showed that 80, 55, and 9% of *A. solani* isolates were resistant boscalid, penthiopyrad, and fluopyram, respectively (Miles et al., 2013). A recent study conducted in *A. solani* on potato demonstrated that single-point mutations exist in the SdhB (H278Y, H278R), SdhC (H134R), and SdhD (H133R and D123E) subunits (Mallik et al., 2014). The H278Y is usually associated with very high boscalid and high penthiopyrad resistance and, the H278R is associated with moderate boscalid and moderate penthiopyrad resistance. The H134R is usually associated with high boscalid and very high penthiopyrad resistance and the H133R and D123E mutations convey very high boscalid resistance and moderate penthiopyrad resistance. All of these mutations convey some level of resistance to both boscalid and penthiopyrad, but none of the mutations express resistance to

fluopyram (Mallik et al., 2014). Furthermore, our research group discovered boscalid resistant isolates from New Mexico, Illinois, Michigan, and Washington in 2013. These *in vitro* trials results suggest that resistance to boscalid is widespread in all major potato producing areas in the United States, due to increased selection pressure caused by heavy usage in the field. As the early blight pathogen is also expressing insensitivity to other SDHI fungicides such as penthiopyrad and fluxapyroxad, there is potential for cross-resistance among fungicides of the SDHI class (Gudmestad et al., 2013). Therefore, it is likely that the increased use of fluopyram will place significant selection pressure on the early blight pathogen, and eventually, resistance may develop to fluopyram.

Resistance to DMI fungicides

The sterol-biosynthesis inhibiting (SBI) fungicides inhibit fungal cell membrane development by preventing ergosterol biosynthesis and triazoles are classified as a chemical group within the DMI class of fungicides (FRAC, 2015). The DMIs have great intrinsic activity due to their post-infection activity against fungal plant pathogens (Wong and Midland, 2007). Specifically, the DMIs inhibit the demethylation of lanosterol by the cytochrome P450 lanosterol 14α-demethylase gene (CYP51A1), as well as possibly the C-22 desaturase site (Brent and Holloman, 2007a). Since the 1980s, resistance problems with the use of DMIs have been reported in several phytopathogenic fungi. DMI resistance development is quantitative, and the fungi is thought to acquire several mutations over time to overcome the fungicide (Brent and Holloman, 2007b). This pattern contrasts the qualitative change of populations observed for other site-specific fungicides of QoI and SDHI. Despite their site-specific mode of action, DMI triazole fungicides are considered to be at medium risk and involve a multi-step process for developing resistance (FRAC, 2015). The buildup of resistance is slow due to the polygenic

control of resistance and the high fitness penalty of the resistant strains (Karaoglanidis et al., 2000).

The first report of DMI resistance was in the barley and cucumber powdery mildew pathogens of *E. graminis f. sp. hordei* and *Sphaerotheca fuligenea*, respectively, in the early 1980s (Russell, 2005). Since then, field resistance to DMI fungicides have developed in 25 phytopathogenic fungi, such as *V. inaequalis* (Stanis and Jones, 1985), *Uncinula necator* (Delye et al., 1997), *C. beticola* (Karaoglanidis et al., 2000), *F. graminiearum* (Yin et al., 2009), and *B. cinerea* (FRAC, 2013). The main mechanisms of DMI resistance are point mutations in the *CYP51* gene, increased amounts of CYP51 protein due to overexpression of the *CYP51* gene, and active transportation of fungicides to the outside of fungal cells (Tateishi et al., 2010). Replacement of tyrosine with phenylalanine at position 136 (Y136F) was discovered in the same gene, which decreases binding site affinity, and, therefore, express fungicide resistance in *U. necator* and *E. graminis* (Delye et al., 1997). Overexpression of the *CYPA1* gene in *V. inaequalis* results in high levels of 14α-demethylase production, which allows the fungus to overcome the effects of the fungicides (Schnabel and Jones, 2001). DMI resistance is also attributed to overexpression of genes that are involved in ATP-binding cassette (ABC) transporter modulators (Stergiopoulos and De Waard, 2002). Although DMIs differ in intrinsic activity, cross-resistance has been reported between compounds of the chemical class in several pathogens (Gisi et al., 2000). Currently, little is known about DMI resistance in *A. solani* and *A. alternata*.

Resistance to AP fungicides

The AP fungicides pyrimethanil, cyprodinil, and mepanipyrim were first introduced in the mid-1990's. Since then, AP fungicides have been registered for control of gray mold on vegetable crops worldwide (Leroux et al., 1999). These chemistries have been rated by FRAC as

having a medium risk of resistance development. However, resistance to AP fungicides has been reported in field isolates of *B. cinerea* of many crops (Amiri et al., 2013; Leroux et al., 1999; Myresiotis et al., 2007), and *V. inaequalis* and *Penicillium expansum* of apple (FRAC 2013; Xiao et al., 2011). Different phenotypes of AP resistance have been detected, with resistance levels varying from low to very high (Caiazzo et al., 2014; Leroux et al., 1999; Myresiotis et al., 2007). Cross-resistance in *B. cinerea* has also been reported among the three fungicides within the AP class (Hilber and Shuepp, 1996; Latorre et al., 2002). A study conducted in Idaho in 2010, reported that 19 and 11% of *A. solani* and *A. alternata* isolates, respectively, demonstrated resistance to pyrimethanil (Fairchild et al., 2013). Despite this report, prevalence and impact of pyrimethanil resistance in a diverse pathogen population is yet to be discovered. The specific objectives of the study were as follows:

1. Compare of conidial germination to mycelial growth for assessing sensitivities of *Alternaria solani* and *Alternaria alternata* to SDHI fungicides.
2. Determine the *in vitro* fungicide sensitivity of *Alternaria* spp. isolates to difenoconazole, metconazole, and pyrimethanil.
3. Determine the effect of *in vitro* reduced-sensitivity of *A. solani* to pyrimethanil on disease control.

MATERIALS AND METHODS

Collection and Maintenance of Isolates

A. solani (Appendix A) and *A. alternata* (Appendix B) isolates that were collected before 2012 were obtained from long-term cryogenic storage (Gudmestad et al., 2013; Holm, 2000; Pasche et al., 2004). Isolates collected after 2012 were obtained from leaf and tuber samples that were submitted to our laboratory during the growing seasons from potato production areas across the nation, including North Dakota, Minnesota, Texas, Nebraska, Michigan, New Mexico, Wisconsin, Colorado, Illinois, Washington, and Idaho (Appendix A and B).

The plant sections were surface sterilized in a 10% bleach solution for 1 min and then rinsed in sterile, distilled water. Early blight and brown spot lesions from leaf samples were transferred to 1.5% agar media (15 g agar and 1000 ml distilled water) and 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 using a sterile glass needle, to a petri plate containing clarified V8 medium (CV-8) (Appendix C) amended with 50 mg/ml ampicillin. Isolates were incubated at 22 ± 2 °C under 24 h fluorescent light for a week and examined for the presence of *A. solani* or *A. alternata* conidia (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 centrifuge tubes. The loosely capped tubes were placed in a closed container with silica gel for 2 to 3 days to remove moisture from the media, and then capped, sealed with Parafilm, and preserved at -80 °C in an ultra-freezer. Herbarium specimens were also made for each tissue sample from which *A. solani* and *A. alternata* isolates were obtained. The specimens were obtained by pressing tissue samples between cardboard and placing the press in a drying chamber for 5 to 7 days. After drying, the specimens were placed in large envelopes and stored at room temperature.

Comparison of Conidial Germination Inhibition to Mycelial Growth Inhibition

In vitro sensitivity of *Alternaria alternata* to fluopyram

A study was performed to compare the *in vitro* sensitivity of *A. alternata* to SDHI fungicides using a conidial germination inhibition assay and a mycelial growth inhibition assay (Avenot and Michailides, 2007). Fifty *A. alternata* isolates collected from 1999 to 2002 with no exposure to SDHI fungicides, were obtained from long-term cryogenic storage (Table 1). Isolates were grown in quarter-strength potato dextrose agar (Potato dextrose broth, 10 g; agar, 12 g; and distilled water, 1000 ml) for seven days at 22 ± 2 °C under 24 h fluorescent light (Avenot and Michailides, 2007). In the *in vitro* poison agar conidial germination inhibition method, a glass rod was used to scrape conidia from the agar surface using ddH_2O (Pasche et al., 2004). The conidial concentration was adjusted by dilution with distilled water to 10^4 conidia/ml using a hemocytometer, and 100 µl was added to the surface of the fungicide amended media. Media containing 2% laboratory grade agar (A360-500 Fisher Scientific, Pittsburgh, PA) was amended with the technical formulation of fluopyram (97.78% a.i.; Bayer CropScience, Raleigh, NC) dissolved in acetone to reach final concentrations of 0, 0.01, 0.1, 1, and 10 mg/ml. The final concentration of acetone in the media was 0.1% by volume. Salicylhydroxamic acid (SHAM) (Sigma Chemical Company, St. Louis, MO) which has been previously determined to inhibit the alternative respiratory pathway (Pasche et al., 2004), was dissolved in methanol and added at 100 mg/ml to the media. The final concentration of methanol in the media was 0.1% by volume.

Table 1. Collection information and mean *in vitro* sensitivity of *Alternaria alternata* to fluopyram

Year	Isolate	Location	EC_{50} (µg/ml)	
			Conidial Germination Inhibition	**Mycelial Growth inhibition**
1999	121-2	Clovis, NM	3.31	2.17
1999	121-3	Clovis, NM	3.75	2.78
1999	122-1	Clovis, NM	3.67	3.09
1999	122-3	Clovis, NM	2.07	2.39
1999	123-2	Dalhart, TX	3.05	2.95
1999	123-6	Dalhart, TX	3.00	1.60
1999	125-1	Clovis, NM	3.02	2.66
1999	125-2	Clovis, NM	2.94	0.66
1999	128-1	Dalhart, TX	3.29	1.73
1999	128-3	Dalhart, TX	3.56	1.62
1999	128-5	Dalhart, TX	2.92	1.59
1999	147-1B	Dalhart, TX	3.46	1.66
1999	147-2	Dalhart, TX	2.90	3.71
1999	147-3	Dalhart, TX	3.78	4.49
1999	147-6	Dalhart, TX	3.52	1.28
1999	147-8	Dalhart, TX	4.10	2.57
1999	154-1	Rexburg, ID	3.12	3.69
1999	178-2	Dalhart, TX	3.34	2.85
1999	183-3	Dawson, ND	3.60	1.22
1999	187-2	Park Rapids, MN	3.98	1.38
1999	189-1	Banner. NE	3.51	2.19
1999	209-4	Alamosa, CO	1.64	3.35
1999	218-5	Karlstad, MN	3.64	2.31
1999	230-1	Park Rapids, MN	2.54	4.12
2000	245-3	Olton, TX	3.90	1.86
2000	247-8	Dalhart, TX	3.40	2.66
2000	302-1	Dawson, ND	3.83	1.68
2000	306-1	Dawson, ND	2.46	2.94
2000	310	Rupert, ID	4.09	1.38
2000	314	Rupert, ID	3.61	2.13
2000	336-1	St. Thomas, ND	3.39	3.66
2000	364-1	Dawson, ND	3.21	4.35
2000	371	Oakes, ND	3.32	1.69
2000	396	Oakes, ND	1.53	4.17
2000	435	Rupert, ID	2.32	3.03
2000	444	Larimore, ND	2.79	2.99
2000	451-1	Menomenie, WI	3.09	4.01
2000	479-4	Dawson, ND	2.45	2.15
2001	527-1A	Dawson, ND	3.39	2.85
2001	527-2A	Dawson, ND	3.49	3.39
2001	547-5	Dawson, ND	2.84	3.31
2002	596-2	Browerville, MN	3.54	3.50

Table 1. Collection information and mean *in vitro* sensitivity of *Alternaria alternata* to fluopyram (continued)

Year	Isolate	Location	EC_{50} (μg/ml)	
			Conidial Germination Inhibition	Mycelial Growth Inhibition
2002	604-1	Columbus, NE	3.57	2.20
2002	613-1SE	Dawson, ND	2.31	2.83
2002	613-5NW	Dawson, ND	2.49	3.60
2002	613-9SW	Dawson, ND	2.35	4.04
2002	613-10NE	Dawson, ND	2.66	3.09
2002	618-1	Dawson, ND	2.37	1.69
2002	649-2	Dawson, ND	2.95	4.06
2002	762-1	Dalhart, TX	5.03	2.16
		Standard deviation=	0.66	0.96

After incubation at 24 ± 2 °C in darkness for 16 h in a Precision® incubator (GCA Corporation, Chicago, IL), the percentage of spore germination (50 conidia for each treatment) was estimated using a compound microscope at ×100 magnification (Avenot and Michailides, 2007). A conidium was considered germinated if one germ tube was at least as long as the conidium, or multiple germ tubes developed from a single conidium (Pasche et al., 2004). In the *in vitro* poison agar mycelial growth inhibition method, a 5 mm mycelial plug from the margin of a 7-day-old *A. alternata* culture was placed on the fungicide amended media. After incubation at 24 ± 2 °C in darkness for seven days, the mycelial growth (colony diameter) of each isolate was measured in two perpendicular directions, with the original mycelial plug diameter (5 mm) subtracted from this measurement.

In vitro sensitivity of *Alternaria solani* to boscalid

Fifty seven *A. solani* isolates collected in 1998 and 2001 with no exposure to boscalid, were obtained from long-term cryogenic storage (Table 2). Isolates were grown in CV-8 medium for 7 to 14 days under 24 h fluorescent light at 22 ± 2 °C (Pasche et al., 2004). In the *in vitro* poison agar conidial germination inhibition method, the conidial concentration was adjusted by

dilution with distilled water to 2×10^4 conidia/ml, and 150 µl was added to the surface of the fungicide amended media. Media containing 2% laboratory grade agar was amended with the technical formulation of boscalid (99% a.i.; BASF Corporation, Research Triangle Park, NC) dissolved in acetone, to reach final concentrations of 0, 0.01, 0.1, 1, and 10 µg/ml and SHAM was added at 100 µg/ml to the media. After incubation at 21 ± 2 °C under continuous light for 4 h, the percentage of spore germination (50 spores for each treatment) was estimated by using a compound microscope at ×100 magnification. In the *in vitro* poison agar mycelial growth inhibition method a 5 mm mycelial plug from the margin of a 4-day-old *A. solani* culture was placed on the fungicide amended media. After incubation at 24 ± 2 °C in darkness for 7 days, the mycelial growth of each isolate was measured as described previously.

Table 2. Collection information and mean *in vitro* sensitivity of *Alternaria solani* to boscalid

Year	Isolate	Location	EC_{50} (µg/ml)	
			Conidial Germination Inhibition	Mycelial Growth Inhibition
1998	1-1	Dalhart, TX	1.21	1.92
1998	3-1	Minden, NE	0.48	1.98
1998	6-1	Park Rapids, MN	0.80	4.43
1998	11-1	Columbus, NE	0.92	0.91
1998	12-1	Minden, NE	0.44	2.93
1998	12-3	Minden, NE	0.50	1.86
1998	13-1	O'Neil, NE	0.56	3.32
1998	14-1	Minden, NE	1.28	1.75
1998	14-3	Minden, NE	0.29	1.76
1998	17-1	Hastings, MN	0.31	3.13
1998	22-1	Staples, MN	0.47	2.98
1998	30-1	Buxton, ND	0.74	4.19
1998	31-1	O'Neil, NE	0.30	4.34
1998	31-4	O'Neil, NE	0.77	4.73
1998	32-1	Park Rapids, MN	1.28	4.21
1998	33-1	Park Rapids, MN	0.80	3.94
1998	37-1	O'Neil, NE	0.43	2.74
1998	37-4	O'Neil, NE	1.01	3.07
1998	38-1	Minden, NE	0.19	2.24
1998	38-4	Minden, NE	0.53	1.44
1998	40-1	Watertown, SD	0.46	2.58
1998	68-1	Shelley, ID	0.6	2.38
1998	83-1	Hamer, ID	0.97	1.62
1998	88-1	Hancock, WI	0.52	2.47
2001	521-1	O'Neil, NE	0.56	5.55
2001	526-3	O'Neil, NE	0.38	3.38
2001	528-2	O'Neil, NE	0.44	3.40
2001	528-3	O'Neil, NE	0.49	2.58
2001	532-2	O'Neil, NE	0.29	3.91
2001	535-2	O'Neil, NE	0.21	2.18
2001	538-2	O'Neil, NE	0.34	2.09
2001	547-1	Dawson, ND	0.11	3.75
2001	547-2	Dawson, ND	0.23	3.30
2001	547-4	Dawson, ND	0.22	3.17
2001	549-1	Dawson, ND	0.32	1.62
2001	549-2	Dawson, ND	0.11	2.14
2001	549-3	Dawson, ND	0.72	2.45
2001	572-1	Minden, NE	0.35	2.42
2001	574-1	Columbus, NE	0.55	4.92
2001	574-3	Columbus, NE	0.13	3.09
2001	577-1	Minden, NE	0.28	2.51
2001	578-1	Minden, NE	0.18	2.67

Table 2. Collection information and mean *in vitro* sensitivity of *Alternaria solani* to boscalid (continued)

Year	Isolate	Location	EC_{50} (μg/ml)	
			Conidial Germination Inhibition	**Mycelial Growth Inhibition**
2001	580-4	Columbus, NE	0.27	3.63
2001	583-2	Dawson, ND	0.89	3.90
2001	583-3	Dawson, ND	0.49	4.30
2001	584-6	Dawson, ND	0.14	4.18
2001	585-1	Dawson, ND	0.33	3.86
2001	586-1	Browerville, MN	0.21	3.49
2001	586-2	Browerville, MN	0.31	3.83
2001	587-3	Browerville, MN	0.30	2.89
2001	587-4	Browerville, MN	0.18	4.49
2001	587-5	Browerville, MN	0.43	3.12
2001	588-1	Kearney, NE	0.33	4.49
2001	588-2	Kearney, NE	0.30	3.24
2001	589-1	Kearney, NE	0.21	0.47
2001	589-2	Kearney, NE	0.40	5.40
2001	590-1	Pettibone, ND	0.33	4.41
		Standard deviation=	0.29	1.11

***In Vitro* Baseline Sensitivity of *Alternaria solani* and *Alternaria alternata* to Difenoconazole and Metconazole**

Fifty *A. alternata* isolates (Table 1) and 57 *A. solani* isolates (Table 2) with no exposure to DMI fungicides also were used for this objective. DMI sensitivity was determined via mycelial growth assays conducted as described previously in evaluations of *D. bryoniae* sensitivity to DMI fungicides (Keinath and Hansen, 2013). Working cultures were transferred onto CV-8 medium and incubated under 24 h fluorescent light at 22 ± 2 °C After four days, 5 mm agar plugs were excised from the leading edge of growth and inverted onto 60 mm petri plates containing 2% laboratory grade agar amended with technical formulations of difenoconazole (95% a.i.; Syngenta Crop Protection, Greensboro, NC) and metconazole (99% a.i.; Valent U.S.A. Corporation, Walnut Creek, CA) dissolved in acetone to reach final

concentrations of 0, 0.01, 0.1, 1, and 10 µg/ml. After incubation at 25 ± 2 °C in darkness for seven days, the mycelial growth of each isolate was measured as described previously.

In Vitro Baseline Sensitivity of _Alternaria solani_ and _Alternaria alternata_ to Pyrimethanil

Fifty *A. alternata* isolates (Table 1) and 57 *A. solani* isolates (Table 2) with no exposure to pyrimethanil also were used for this objective. Fungicide sensitivity was determined via a mycelial growth assay on a synthetic medium containing L-asparagine (asp-agar) as described previously for evaluating *B. cinerea* sensitivity to group 9 fungicides (Hilber and Schüepp, 1996). Media containing asp-agar (Appendix D) were amended with technical grade pyrimethanil (95% a.i.; Bayer CropScience, Raleigh, NC) dissolved in acetone to reach final concentrations of 0, 0.1, 1, and 10 µg/ml (Hilber and Schüepp, 1996).

Sensitivity of Non-Baseline Isolates of _Alternaria solani_ to Difenoconazole, Metconazole, and Pyrimethanil

Two hundred forty five *A. solani* isolates (Appendix A) collected in 2010, 2011, 2012, 2013, and 2014 were used for *in vitro* fungicide sensitivity screening. The isolates were selected based on their geographic location and were primarily obtained from samples submitted by potato growers. Fungicide sensitivity was determined as described above on DMI-amended 2% water agar at concentrations of 0, 0.01, 0.1, 1, 10, and 100 µg/ml, and pyrimethanil-amended asp agar at 0, 0.1, 1, 10, and 100 µg/ml. Additionally, 109 *A. alternata* isolates (Appendix B) collected in 2011, 2013, and 2014 alswere tested for a shift in sensitivity to pyrimethanil.

Effect of Reduced-Sensitivity of _Alternaria solani_ to Pyrimethanil on Disease Control

The significance of the *in vitro* shift in sensitivity of *A. solani* to pyrimethanil on disease control was determined under greenhouse conditions as previously described (Pasche et al., 2004; 2005). A subset of six isolates of *A. solani* (Table 3) was tested for *in vivo* sensitivity

based upon *in vitro* pyrimethanil sensitivity. Two sensitive and four pyrimethanil reduced-sensitive isolates were used. All isolates were recovered from long-term storage, maintained, and conidia were harvested.

Table 3. *Alternaria solani* isolates tested *in vivo* for sensitivity to pyrimethanil

Isolate	Origin	Cultivar	Collection Year	EC_{50}[#] (μg/ml)	Sensitive/ reduced-sensitive[†]
13-1	O'Neil, NE	Russet Norkotah	1998	0.52	S
1179-3	Pettibone, ND	Unknown	2010	0.75	S
1168-3	Acequia, ID	Unknown	2010	1.57	RS
1184-14	Wray, CO	FL 1867	2011	3.70	RS
1191-13	Wadena, MN	Unknown	2011	28.26	RS
1332-6	Dalhart, TX	Russet Norkotah	2013	2.42	RS

[#] EC_{50} values were obtained for pyrimethanil from the *in vitro* assessment.
[†] Isolates were characterized as sensitive (S) or reduced-sensitive (RS) based on RF values (S = ≤4, RS = >4)

The *in vivo* sensitivity assay was conducted as a 24-h preventive test. Pyrimethanil was applied 24 h prior to inoculation in the greenhouse using tomato plants, cv. Orange Pixie VFT Hybrid (Tomato Growers Supply Company, Fort Myers, FL). This cultivar was used because of its susceptibility to early blight and compact size compared with potato plants. This allowed for adequate replication for evaluating multiple fungicide concentrations across several *A. solani* isolates. Three tomato seeds were sown in each 10 cm^3 plastic pots, containing Sunshine Mix LC1 (Sun Gro Horticulture Inc., Bellevue, WA) and after 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, they were treated with commercial formulation of pyrimethanil (37.4% a.i.; Scala® 400 SC, Bayer). Ten-fold fungicide concentrations of the active ingredient were applied to the plants (0, 0.1, 1, 10, and 100 μg/ml) to obtain a dose response curve. Fungicide was applied using a Generation II Research Sprayer (Devries Manufacturing, Hollandale, MN) at approximately 400 kPa. 24 h after fungicide application, plants were

inoculated using 50 ml of 2.0×10^5 conidia/ml suspension, produced from 10- to 12-day-old-cultures of *A. solani* maintained on CV-8 medium for 7 to 14 days under 24 h fluorescent light at 22 ± 2 °C. A Preval paint-spray gun (Preval Sprayer Division, Precision Valve Corporation, Yonkers, NY) was used for inoculation and inoculated plants were kept in individual humidity chambers (Phytotronic Inc.; 1626D) for 24 h at >95% RH and 22 ± 2 °C. Plants were transferred to confinement chambers (plastic chambers with an open ceiling) on greenhouse benches to avoid cross contamination among *A. solani* isolates and were maintained at 25 ± 2 °C with daily application of water. Early blight disease severity was rated visually at 6, 9, and 12 days post inoculation (DPI) by estimating percent infected leaf area of the first three true leaves (three subsamples) and recorded as percentage diseased tissue. Two samples (two plants per pot) and three replications (three pots) were tested for each isolate × fungicide concentration. The *in vivo* experiment was performed three times.

Statistical Analysis

All *in vitro* experiments were performed twice using a completely random design with two replicates for each fungicide concentration. To determine the EC_{50} (Effective concentration at which the fungal growth will be inhibited by 50%) value for each isolate, the percentage reduction in conidial germination or mycelial growth relative to the non-treated control was calculated (Pasche et al., 2004). These data were regressed against the $\log_{10}$ fungicide concentration and the EC_{50} value was determined by interpolation of the 50% intercept using the Statistical Analysis System (SAS Institute Inc., Cary, NC). The experiments were analyzed separately, and the *F*-test was used to test for homogeneity of variance among experiments. In all *in vitro* studies involving *A. alternata*, the coefficient of variability (standard error/mean) of log-10 transformed EC_{50} values among all experimental repeats was calculated as a measure of assay

reproducibility (Thomas et al., 2012). In all *in vitro* studies involving *A. solani*, control isolates 13-1, a wild-type *A. solani* isolate, and 526-3, a QoI resistant isolate, were used in each trial as internal controls to determine reproducibility of the assay. Assay reproducibility calculations were applied to the internal controls (Wong and Wilcox, 2002). The assay reproducibility calculations produced a mean EC_{50} value, a coefficient of variance, and approximate bounds for the 95% confidence interval for each of the control. These bounds were approximated because the Land's Coefficients had to be estimated. Trials in which the EC_{50} values of the internal controls are within the 95% confidence interval were included in further statistical analyses. Correlation analysis ($\alpha = 0.05$) was performed using Pearson correlation coefficients to compare *in vitro* fungicide EC_{50} values for both baseline and 2010-2014 *A. solani* and *A. alternata* isolates.

All *in vivo* experiments were split-plot randomized complete block designs with *A. solani* isolates as the main plot and fungicide concentrations as subplots. For each isolate, at all fungicide concentrations, disease severity data were transformed to percent disease control using the formula [1 – (% diseased tissue / % diseased tissue in non-treated plants) × 100]. Levene's Test was conducted to test for homogeneity of variance among three independent experiments (Milliken and Johnson, 1992). The analysis of variance (ANOVA) was performed separately for isolate × fungicide group combination at each fungicide concentration using SAS and *t*-tests were used on the combined data to detect differences at each fungicide concentration. Area under the disease progress curve (AUDPC) for dose response curves were calculated to determine if there was a significant difference in disease control provided by pyrimethanil in controlling sensitive and reduced-sensitive isolates (Shaner and Finney, 1977).

RESULTS

Comparison of Conidial Germination Inhibition to Mycelial Growth Inhibition

In vitro sensitivity of *Alternaria alternata* to fluopyram

F-tests were conducted on both conidial germination and mycelial growth inhibition *in vitro* fungicide sensitivity experiments and it was determined that experimental variances were homogenous in both fungicide assays. There was no significant difference among trials in conidial germination (P = 0.4293) or mycelial growth (P = 0.4998) sensitivity testing, so experiments were combined for each inhibition method, and mean EC_{50} values were calculated for each isolate (Appendix E). The overall mean EC_{50} value of the 50 *A. alternata* isolates tested for conidial germination inhibition was 3.17 μg/ml, and isolate sensitivity ranged from 1.53 to 5.03 μg/ml (Table 4). Mean EC_{50} value of the *A. alternata* isolates tested for mycelial growth inhibition was 2.67 μg/ml, and isolate sensitivity ranged from 0.66 to 4.49 μg/ml (Table 4).

Coefficients of variation of $\log_{10}$-transformed EC_{50} values of individual isolates among experimental repeats were 2% to 18% for conidial germination inhibition and 3% to 20% for mycelial growth inhibition *in vitro* assays (Table 4). The coefficient of variation is less than 20% for both the *in vitro* assays tested, which indicates that the $\log_{10}$-transformed EC_{50} values of individual isolates were consistent among the experimental repeats, proving the reproducibility of the assay.

Table 4. Range, mean, median EC_{50} values, and coefficient of variability based on log_{10}-transformed EC_{50} values of isolates of *Alternaria alternata* for each *in vitro* method in determining fluopyram sensitivity

Method	Trial	EC_{50} (µg/ml) Range	Mean	Median	Coefficient of Variability[#]
Conidial germination	1	1.39-4.84	3.17	3.27	…
	2	1.63-5.23	3.19	3.22	…
	Combined	1.53-5.03	3.17	3.32	0.02-0.18
Mycelial growth	1	0.76-4.99	2.77	2.79	…
	2	0.56-4.35	2.57	2.56	…
	Combined	0.66-4.49	2.67	2.72	0.03-0.20

[#] Coefficient of variability is the absolute value of (standard deviation of log_{10} EC_{50} values)/ (mean of log_{10} EC_{50} values)

The correlation analysis determined that a significant (P = 0.0122) but weak (r = -0.3512) association exists in conidial germination and mycelial growth EC_{50} values among *A. alternata* isolates in response to fluopyram (Table E3; Fig. 1).

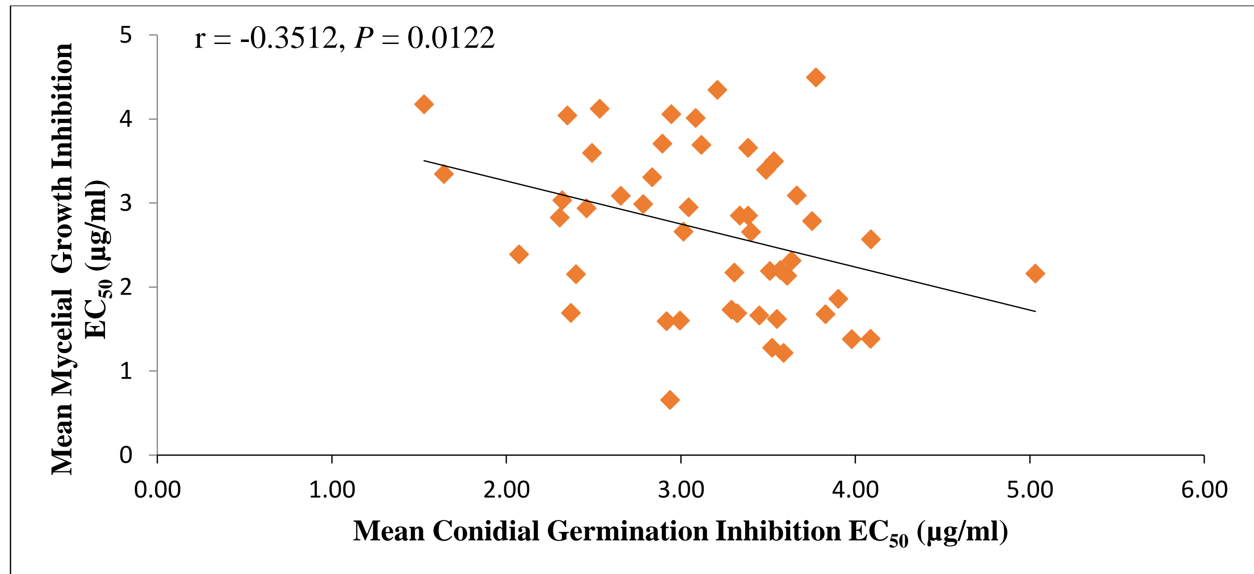

Figure 1. Scatter diagram of the linear correlation between conidial germination inhibition and mycelial growth inhibition of *Alternaria alternata* in response to fluopyram.

In vitro sensitivity of *Alternaria solani* to boscalid

F-tests were conducted for both conidial germination and mycelial growth inhibition *in vitro* fungicide sensitivity experiments and experimental variances were homogenous in both fungicide assays. There were no significant difference among trials for conidial germination (P = 0.2540) or mycelial growth (P = 0.5003) sensitivity testing, so experiments were combined for each inhibition method, and mean EC_{50} values were calculated for each isolate (Appendix F). The overall mean EC_{50} value of the 57 *A. solani* isolates tested for conidial germination inhibition was 0.47 µg/ml, and isolate sensitivity ranged from 0.11 to 1.28 µg/ml (Table 5).

An assay reproducibility analysis was conducted on 13-1, a wild-type *A. solani* isolate, and 526-3, QoI resistant isolate. The internal controls were tested seven times in conjunction with independent experiments. The coefficients of variance for 13-1 and 526-3 were 3% and 3%, respectively (Table 6). The assay reproducibility calculations generated approximate limits for the 95% confidence interval for the two internal controls. Trials in which the EC_{50} values of the internal controls are within the 95% confidence interval were included in further statistical analyses.

The mean EC_{50} value of the *A. solani* isolates tested for mycelial growth inhibition was 3.12 µg/ml, and isolate sensitivity ranged from 0.47 to 5.55 µg/ml (Table 5). An assay reproducibility analysis was conducted on the internal controls and they were tested seven times in conjunction with independent experiments. The coefficients of variance for 13-1 and 526-3 were 13% and 4%, respectively (Table 6). The correlation coefficient comparing EC_{50} values for conidial germination and mycelial growth of *A. solani* isolates in response to boscalid was very low (r = -0.0545), indicating that the association of these two *in vitro* inhibition methods was very weak and not significant (P = 0.6878) (Table F3; Fig. 2).

Table 5. Range, mean, and median EC_{50} values based on $\log_{10}$-transformed EC_{50} values of isolates of *Alternaria solani* for each *in vitro* method in determining boscalid sensitivity

Method	Trial	EC_{50} (µg/ml)		
		Range	Mean	Median
Conidial germination	1	0.10-1.28	0.47	0.39
	2	0.11-1.28	0.46	0.40
	Combined	0.11-1.28	0.47	0.40
Mycelial growth	1	0.48-5.55	3.09	3.05
	2	0.46-5.55	3.14	3.11
	Combined	0.47-5.55	3.12	3.12

Table 6. Reproducibility of the *in vitro* assays for determining boscalid sensitivity of isolates of *Alternaria solani*

Isolate	EC_{50} (µg/ml) conidial germination			EC_{50} (µg/ml) mycelial growth		
	Mean[#]	95% CI[†]	CV[$]	Mean[#]	95% CI[†]	CV[$]
13-1	0.50	(0.43-0.61)	0.03	3.20	(2.78-3.97)	0.13
526-3	0.37	(0.29-0.46)	0.03	3.05	(2.82-3.39)	0.04

[#] Mean EC_{50} value based upon $\log_{10}$ EC_{50} values obtained from seven repeated assays.

[†] 95% confidence interval based upon $\log_{10}$ EC_{50} values.

[$] The coefficient of variance based upon $\log_{10}$ EC_{50} values.

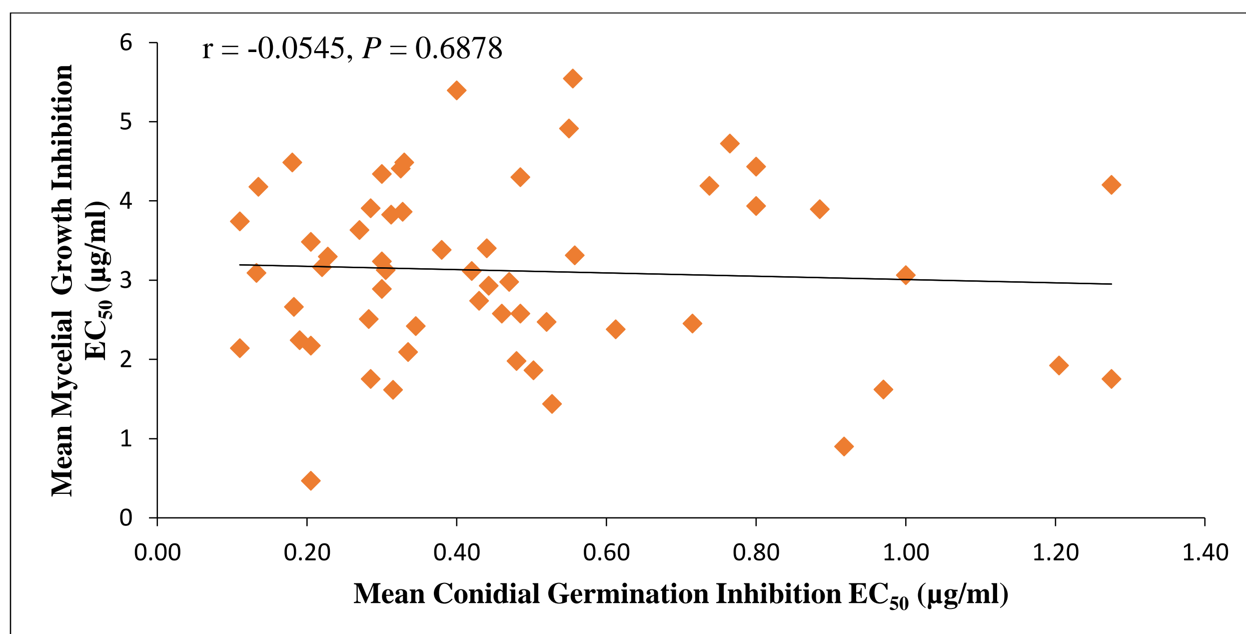

Figure 2. Scatter diagram of the linear correlation between conidial germination inhibition and mycelial growth inhibition of *Alternaria solani* in response to boscalid.

In Vitro Baseline Sensitivity of *Alternaria solani* and *Alternaria alternata* to Difenoconazole and Metconazole

In vitro baseline sensitivity of *Alternaria solani* to DMI fungicides

F-tests were conducted on both difenoconazole and metconazole *in vitro* fungicide sensitivity experiments and experimental variances were homogenous in both fungicide assays. There was no significant difference among trials for difenoconazole ($P = 0.0769$) or metconazole ($P = 0.8536$) sensitivity testing, so experiments were combined for each fungicide, and mean EC_{50} values were calculated for each isolate (Appendix G). EC_{50} values of the *A. solani* isolate sensitivity to difenoconazole and metconazole ranged from 0.02 to 0.30 and 0.04 to 0.18 µg/ml with mean EC_{50} values of 0.09 and 0.09, respectively (Table 7; Fig. 3).

Table 7. Range, mean, and median EC_{50} values based on log_{10}-transformed EC_{50} values of baseline isolates of *Alternaria solani* to DMI fungicides

Method	Trial	EC_{50} (µg/ml)		
		Range	Mean	Median
Difenoconazole	1	0.02-0.28	0.09	0.08
	2	0.02-0.34	0.09	0.08
	Combined	0.02-0.30	0.09	0.08
Metconazole	1	0.04-0.19	0.09	0.09
	2	0.03-0.18	0.09	0.09
	Combined	0.04-0.18	0.09	0.09

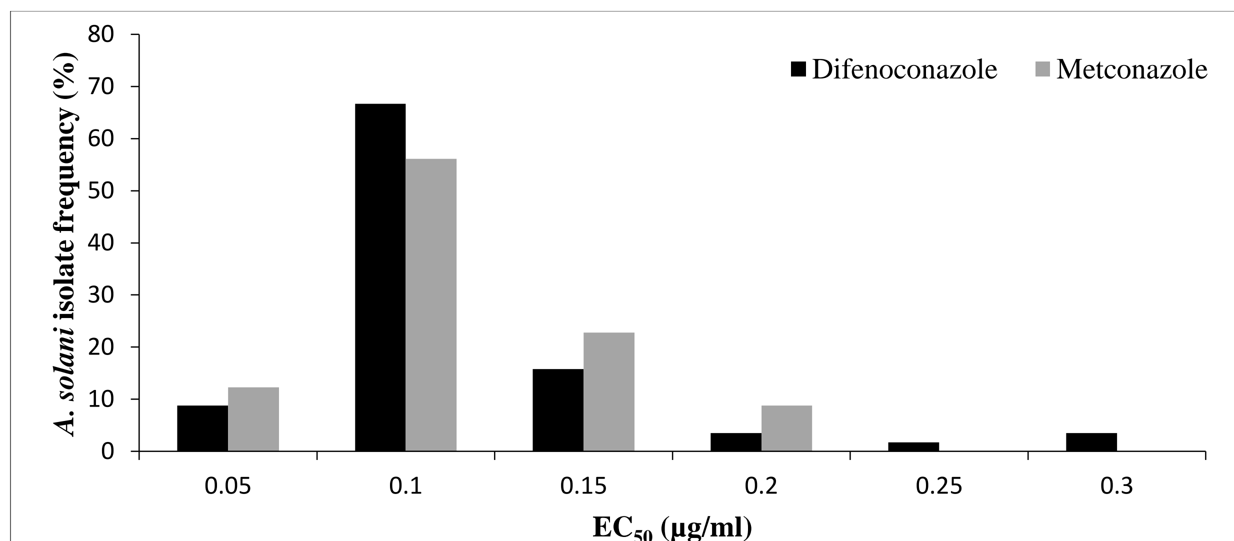

Figure 3. Frequency distribution of sensitivity of 57 baseline *Alternaria solani* isolates to DMI fungicides. Sensitivity is based upon the effective concentration which inhibits mycelial growth by 50% compared to the non-treated control (EC_{50} µg/ml).

An assay reproducibility analysis was conducted and internal controls were tested seven times in conjunction with independent experiments. The coefficients of variance for 13-1 and 526-3 were 1% and 1%, respectively, for difenoconazole sensitivity testing (Table 8). The coefficients of variance for 13-1 and 526-3 were 1% and 1%, respectively, for metconazole sensitivity testing (Table 8). The correlation coefficient comparing EC_{50} values for difenoconazole and metconazole baseline sensitivities of *A. solani* isolates was very low (r = 0.0205), indicating that the association between these two fungicides was very weak and not significant (P = 0.8797) (Fig. 4).

Table 8. Reproducibility of the *in vitro* assays for determining DMI sensitivity of baseline isolates of *Alternaria solani*

Isolate	EC_{50} (µg/ml) difenoconazole			EC_{50} (µg/ml) metconazole		
	Mean#	95% CI†	CV$	Mean#	95% CI†	CV$
13-1	0.10	(0.08-0.12)	0.01	0.15	(0.13-0.17)	0.01
526-3	0.08	(0.06-0.10)	0.01	0.14	(0.12-0.16)	0.01

Mean EC_{50} value based upon log_{10} EC_{50} values obtained from seven repeated assays.
† 95% confidence interval based upon log_{10} EC_{50} values.
$ The coefficient of variance based upon log_{10} EC_{50} values.

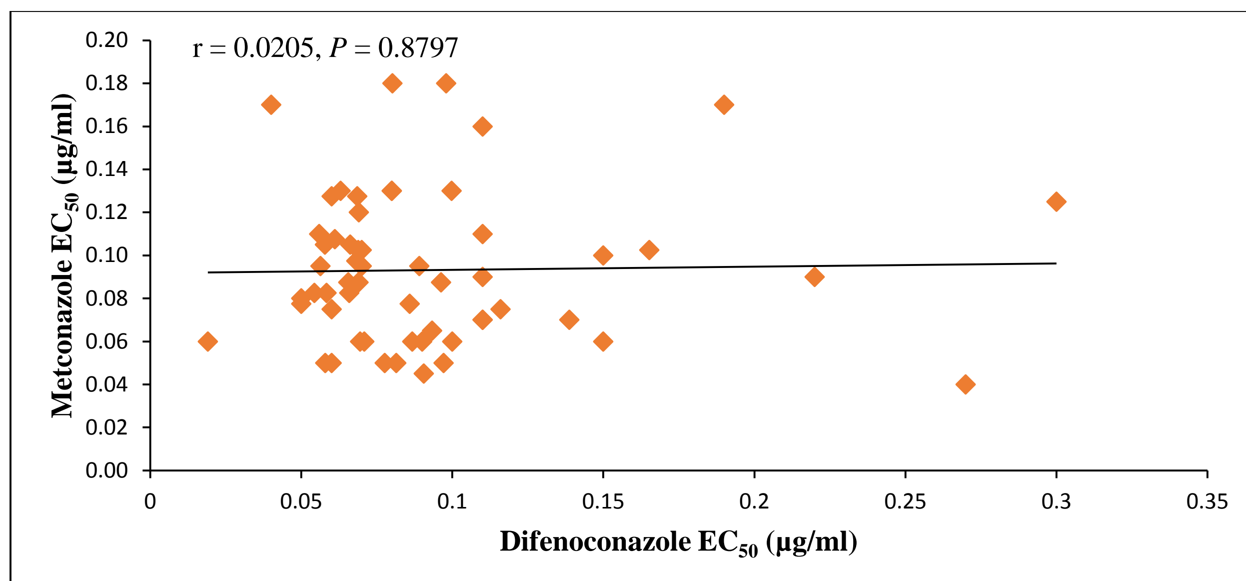

Figure 4. Scatter diagram of the linear correlation between the *in vitro* difenoconazole and metconazole sensitivity of 57 baseline *Alternaria solani* isolates.

<u>*In vitro* baseline sensitivity of *Alternaria alternata* to DMI fungicides</u>

F-tests were conducted on both difenoconazole and metconazole *in vitro* fungicide sensitivity experiments and experimental variances were homogenous in both fungicide assays. There was no significant difference among trials for difenoconazole ($P = 0.7095$) or metconazole ($P = 0.1590$) sensitivity testing, so experiments were combined for each inhibition method, and mean EC_{50} values were calculated for each isolate (Appendix H). EC_{50} values of the *A. alternata* isolate sensitivity to difenoconazole and metconazole ranged from 0.03 to 0.33 and 0.04 to 0.48 µg/ml with mean EC_{50} values of 0.14 and 0.26, respectively (Fig. 5).

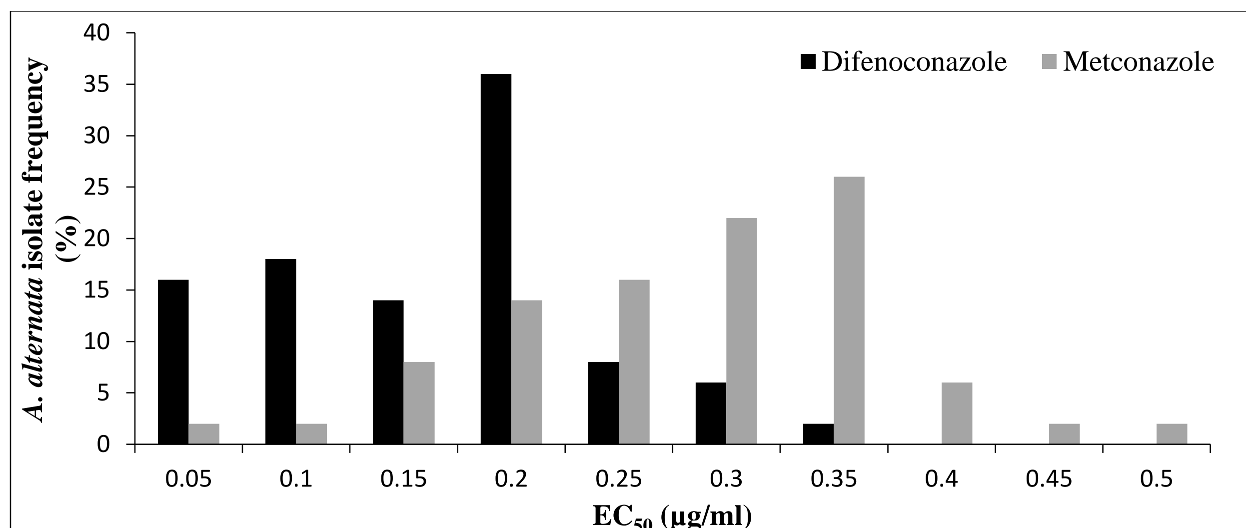

Figure 5. Frequency distribution of sensitivity of 50 baseline *Alternaria alternata* isolates to DMI fungicides. Sensitivity is based upon the effective concentration which inhibits mycelial growth by 50% compared to the non-treated control (EC_{50} µg/ml).

Coefficients of variation of log_{10}-transformed EC_{50} values of individual isolates among experimental repeats were 0% to 18% for difenoconazole, and 0% to 15% for metconazole *in vitro* assays (Table 9). The coefficient of variation is less than 20% for both fungicides tested, which indicates that the log_{10}-transformed EC_{50} values of individual isolates were consistent among the experimental repeats, proving the reproducibility of the assay.

Table 9. Range, mean, median EC_{50} values, and coefficient of variability based on log_{10}-transformed EC_{50} values of baseline isolates of *Alternaria alternata* to DMI fungicides

		EC_{50} (µg/ml)			
Fungicide	**Trial**	**Range**	**Mean**	**Median**	**Coefficient of variability#**
Difenoconazole	1	0.03-0.33	0.14	0.16	…
	2	0.03-0.33	0.14	0.15	…
	Combined	0.03-0.33	0.14	0.16	0-0.18
Metconazole	1	0.04-0.48	0.26	0.27	…
	2	0.04-0.48	0.26	0.28	…
	Combined	0.04-0.48	0.26	0.27	0-0.15

Coefficient of variability is the absolute value of (standard deviation of log_{10} EC_{50} values)/ (mean of log_{10} EC_{50} values)

In contrast to *A. solani*, the correlation analysis disclosed a significant association between EC_{50} values for difenoconazole and metconazole baseline sensitivities of *A. alternata* isolates ($r = 0.7141$, $P < 0.0001$) (Fig. 6).

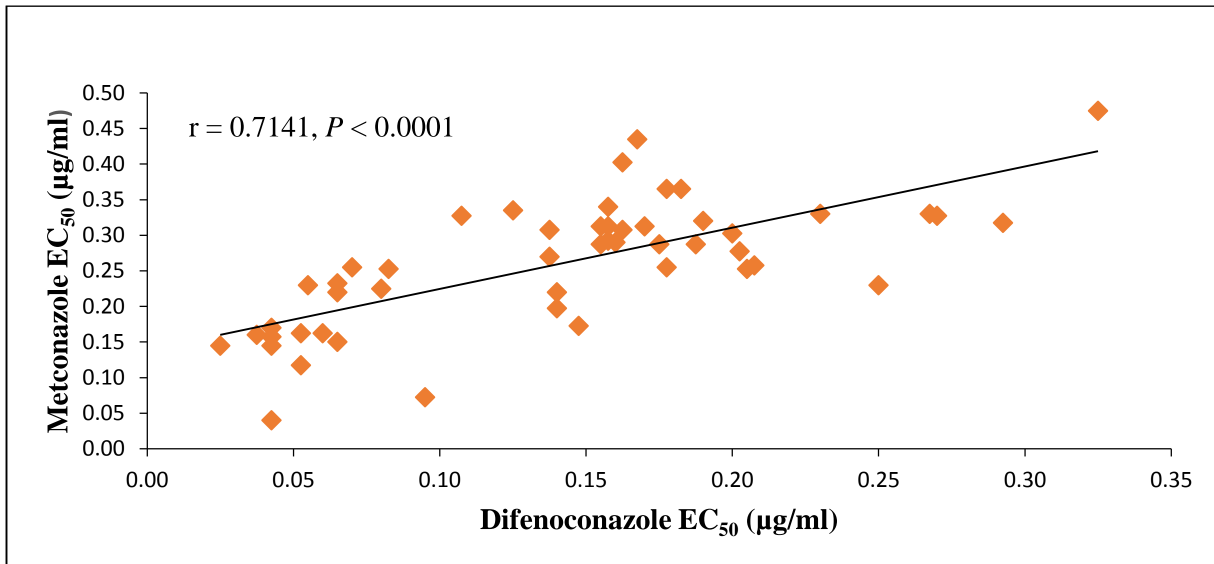

Figure 6. Scatter diagram of the linear correlation between the *in vitro* difenoconazole and metconazole sensitivity of 50 baseline *Alternaria alternata* isolates.

In Vitro Baseline Sensitivity of *Alternaria solani* and *Alternaria alternata* to Pyrimethanil

F-tests were conducted on both *A. solani* and *A. alternata in vitro* fungicide sensitivity experiments and it was determined that experimental variances were homogenous in both fungicide assays. There was no significant difference among trials for *A. solani* ($P = 0.6431$) or *A. alternata* ($P = 0.8000$) sensitivity testing, so experiments were combined for each inhibition method, and mean EC_{50} values were calculated for each isolate (Appendix I). EC_{50} values of the *A. solani* isolate sensitivity for pyrimethanil ranged from 0.35 to 0.58 µg/ml with mean EC_{50} value of 0.44 (Table 10; Fig. 7). EC_{50} values of the *A. alternata* isolate sensitivity for pyrimethanil ranged from 0.15 to 0.42 µg/ml with mean EC_{50} value of 0.35 (Table 10; Fig. 7).

Table 10. Range, mean, median EC_{50} values, and coefficient of variability based on log_{10}-transformed EC_{50} values of baseline isolates of *Alternaria* spp. isolates to pyrimethanil

Pathogen	Trial	EC_{50} (µg/ml)			Coefficient of variability[#]
		Range	Mean	Median	
Alternaria solani	1	0.35-0.58	0.44	0.43	…
	2	0.36-0.58	0.44	0.44	…
	Combined	0.35-0.58	0.44	0.43	N/A
Alternaria alternata	1	0.15-0.42	0.35	0.36	…
	2	0.15-0.42	0.35	0.37	…
	Combined	0.15-0.42	0.35	0.37	0.01-0.18

[#] Coefficient of variability is the absolute value of (standard deviation of log_{10} EC_{50} values)/ (mean of log_{10} EC_{50} values)

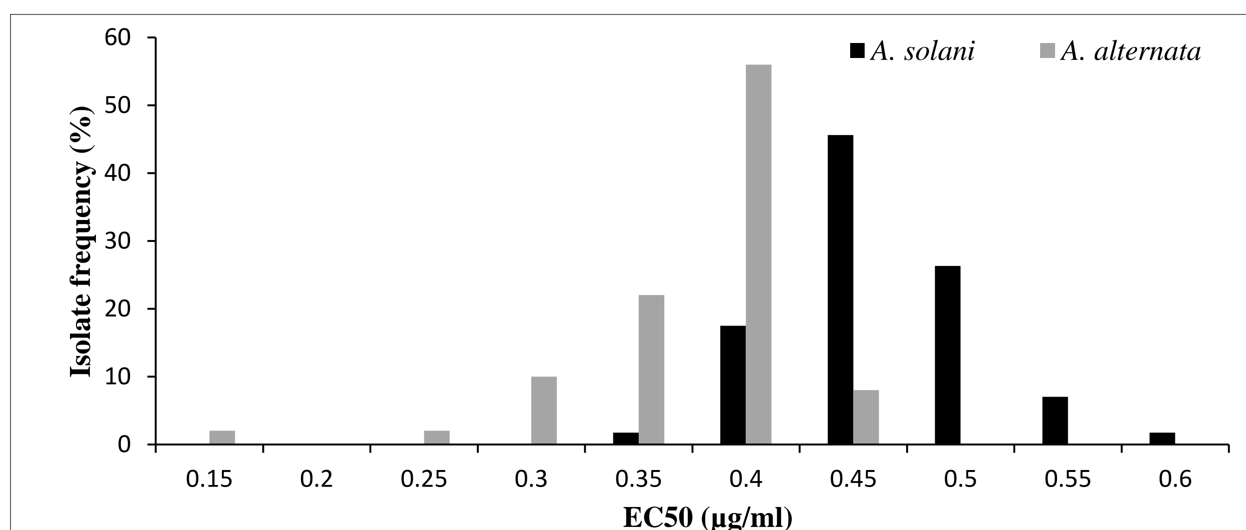

Figure 7. Frequency distribution of sensitivity of baseline *Alternaria* spp. isolates to pyrimethanil. Sensitivity is based upon the effective concentration which inhibits mycelial growth by 50% compared to the non-treated control (EC_{50} µg/ml).

An assay reproducibility analysis was conducted on the internal controls and they were tested seven times in conjunction with independent experiments. The coefficients of variance for 13-1 and 526-3 were 1% and 1%, respectively, for pyrimethanil sensitivity testing (Table 11).

Table 11. Reproducibility of the *in vitro* assays for determining pyrimethanil sensitivity of baseline isolates of *Alternaria solani*

Isolate	Mean#	95% CI†	CV$
13-1	0.52	(0.47-0.55)	0.01
526-3	0.51	(0.48-0.54)	0.01

Mean EC_{50} value based upon $\log_{10}$ EC_{50} values obtained from seven repeated assays.

† 95% confidence interval based upon $\log_{10}$ EC_{50} values.

$ The coefficient of variance based upon $\log_{10}$ EC_{50} values.

Coefficients of variation of $\log_{10}$-transformed EC_{50} values of individual *A. alternata* isolates among experimental repeats were 1% to 18% (Table 10). The coefficient of variation is less than 20%, which indicates that the $\log_{10}$-transformed EC_{50} values of individual isolates were consistent among the experimental repeats, which proves the reproducibility of the assay.

Sensitivity of Non-Baseline Isolates of *Alternaria solani* to Difenoconazole, Metconazole, and Pyrimethanil

Independent analysis of variance of *in vitro* fungicide sensitivity experiments for difenoconazole, metconazole, and pyrimethanil EC_{50} values determined that error variances were homogenous ($P = 0.05$); thus, experiments were combined by individual fungicide. The overall mean fungicide sensitivities of the fifty five 2010 *A. solani* isolates to difenoconazole and metconazole was 0.12 and 0.18 µg/ml, respectively (Fig. 8). Mean EC_{50} values for the 109 isolates from 2011 were 0.13 and 0.19 µg/ml for difenoconazole and metconazole, respectively (Fig. 8). Eight isolates from 2012 had mean EC_{50} values of 0.14 µg/ml and 0.25 µg/ml for difenoconazole and metconazole, respectively (Fig. 8). Mean EC_{50} values for the 58 isolates from 2013 were 0.07 and 0.16 µg/ml for difenoconazole and metconazole, respectively, while 15 isolates from 2014 had mean EC_{50} values of 0.06 µg/ml and 0.10 µg/ml for difenoconazole and metconazole (Fig. 8).

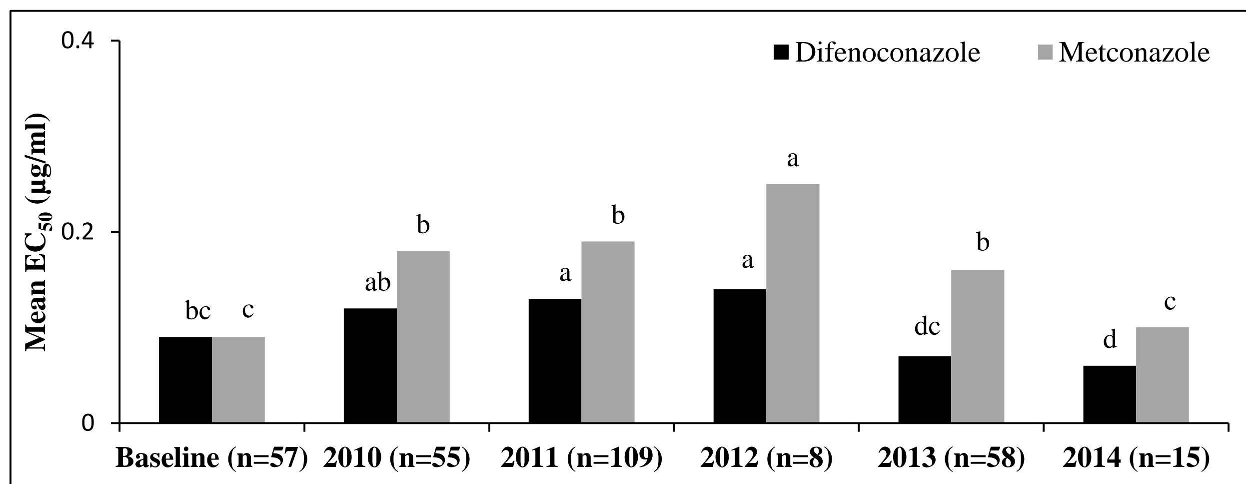

Figure 8. Mean EC_{50} values for *in vitro* isolate sensitivity of *Alternaria solani* to DMI fungicides across years. Within fungicides, columns with the same letter are not significantly different based on Fisher's protected least significant difference at the $P = 0.05$ level.

Individual analysis of *A. solani* isolate EC_{50} values for each fungicide revealed a significant difference between mean EC_{50} value of baseline and fungicide exposed isolates for both difenoconazole and metconazole ($P = 0.0228$, and $P < 0.0001$), respectively (Fig. 8). In this study, the RF values for *A. solani* sensitivity testing were 1.2 and 2.0 for difenoconazole and metconazole, respectively. Isolates collected in 2012 had significantly higher EC_{50} values for metconazole than each of the preceding years, while isolates collected 2012 had the highest EC_{50} value for difenoconazole, and isolates collected in 2014 had the lowest EC_{50} value for difenoconazole sensitivity than isolates collected in other years (Fig. 8). In contrast to the baseline *A. solani* isolates, the correlation analysis disclosed a significant association between EC_{50} values for difenoconazole and metconazole sensitivities of *A. solani* isolates collected from 2010 to 2014 ($r = 0.4962$, $P < 0.0001$) (Fig. 9). The difference may be due to the number of isolates used in the baseline (n = 57) and non-baseline (n = 245) sensitivity testing.

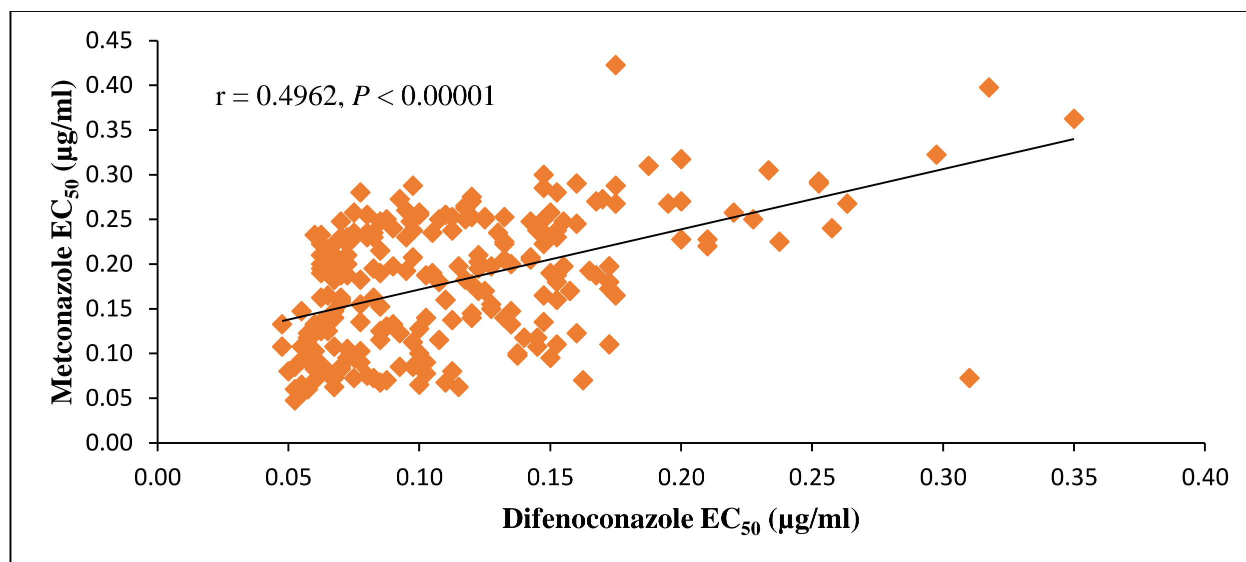

Figure 9. Scatter diagram of the linear correlation between the *in vitro* difenoconazole and metconazole sensitivity of 245 *Alternaria solani* isolates collected from 2010 to 2014.

The overall mean pyrimethanil sensitivity for the 55 *A. solani* isolates collected in 2010 was 0.74 µg/ml (Fig. 10). The mean EC_{50} value for the 109 isolates from 2011 was 0.95 µg/ml, while eight isolates from 2012 had a mean EC_{50} value of 0.50 µg/ml (Fig. 10). The mean EC_{50} value for the 58 isolates from 2013 was 0.54 µg/ml, while 15 isolates from 2014 had a mean EC_{50} value of 0.57 µg/ml (Fig. 10). Although there was no significant ($P = 0.1701$) difference between mean EC_{50} value of baseline and fungicide exposed isolates, six *A. solani* isolates had EC_{50} values greater than mean baseline value by 4-fold (Fig. 10).

Additionally *A. alternata* isolates collected from 2011 to 2014 were tested for pyrimethanil sensitivity with significant difference between mean EC_{50} value of baseline and fungicide exposed isolates ($P < 0.0001$) (Fig. 10). The overall mean pyrimethanil sensitivity for 19 *A. alternata* isolates collected in 2011 was 0.52 µg/ml (Fig. 10). The mean EC_{50} value for the 75 isolates from 2013 was 0.47 µg/ml, while 15 isolates from 2014 had a mean EC_{50} value of 0.61 µg/ml (Fig. 10). In this study, the RF value for *A. alternata* sensitivity testing was 1.4 for pyrimethanil.

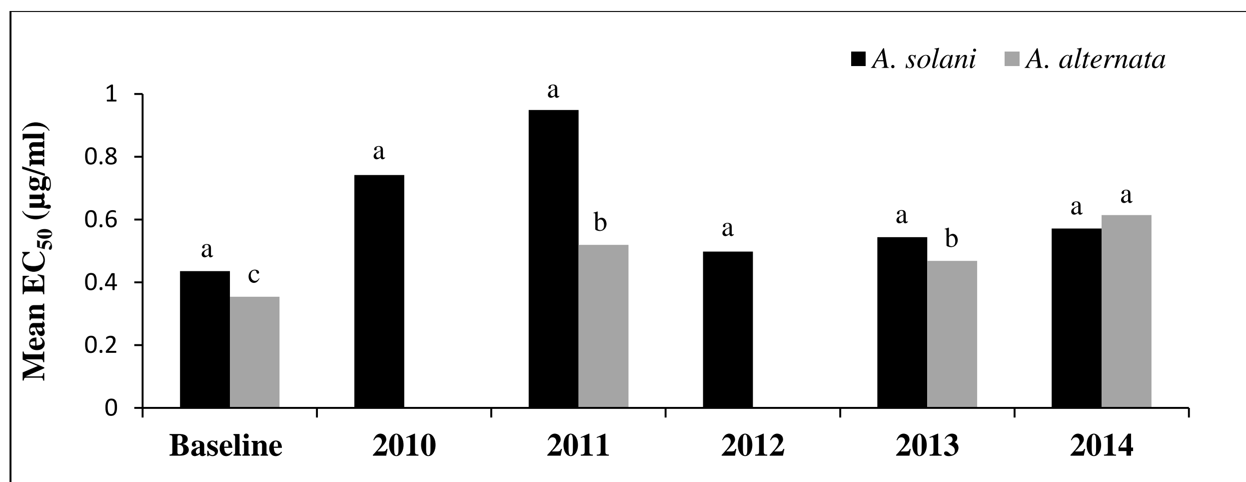

Figure 10. Mean EC_{50} values for *in vitro* isolate sensitivity of *Alternaria solani* and *Alternaria alternata* to pyrimethanil across years. Within species, columns with the same letter are not significantly different based on Fisher's protected least significant difference at the $P = 0.05$ level.

Effect of Reduced-Sensitivity of *A. solani* to Pyrimethanil on Disease Control

Independent analysis of *in vivo* disease control experiments for pyrimethanil determined that variances were homogenous ($P = 0.05$); thus, experiments were combined for further analysis. Based on AUDPC calculations, significant interaction between the main plot (isolate) and subplot factor (fungicide concentrations) ($P < 0.0001$) was observed for percentage disease control of pyrimethanil on *A. solani*-infected greenhouse grown tomato plants. Significant effects ($P < 0.0001$) were also observed for isolate and level of fungicide concentration for percentage disease control. Dose response curves indicate that sensitive *A. solani* isolates (13-1 and 1179-3) were controlled similarly at all fungicide concentrations except 1 µg/ml (Table 12; Fig. 11). Significant differences were observed in the disease control of reduced-sensitive isolates (1168-3, 1184-14, 1191-13, and 1332-6) at concentrations of 0.1, 1, and 10 µg/ml (Table 12; Fig. 11). For all concentrations, disease control of reduced-sensitive isolates of *A. solani* was significantly less than the disease control provided by pyrimethanil on sensitive isolates (Table 12; Fig. 11).

Table 12. Mean *in vivo* percentage disease control of *Alternaria solani* isolates by pyrimethanil as determined in greenhouse assays

Isolate	EC_{50}[#] (µg/ml)	Sensitive/ reduced-sensitive[†]	Pyrimethanil concentration (µg/ml) 0.1	1	10	100	AUDRC[$]
13-1	0.52	S	7.4 a	37.0 a	88.9 a	94.8 a	8854.5 a
1179-3	0.75	S	5.5 ab	32.3 b	89.4 a	96.4 a	8927.4 a
1168-3	1.57	RS	5.0 b	28.2 c	64.7 c	90.7 b	7427.1 c
1184-14	3.70	RS	2.2 c	22.2 d	63.6 c	91.0 b	7355.6 c
1191-13	28.26	RS	3.7 bc	11.5 f	60.9 c	91.1 b	7171.5 d
1332-6	2.42	RS	2.4 c	18.0 e	69.5 b	91.1 b	7932.3 b
$LSD_{P=0.05}$			2.1	3.1	4.1	1.8	171.7

[#] EC_{50} values were obtained for pyrimethanil from the *in vitro* assessment.

[†] Isolates were characterized as sensitive (S) or reduced-sensitive (RS) based on RF values (S = ≤4, RS = >4)

[$]AUDRC= Area under dose response curve

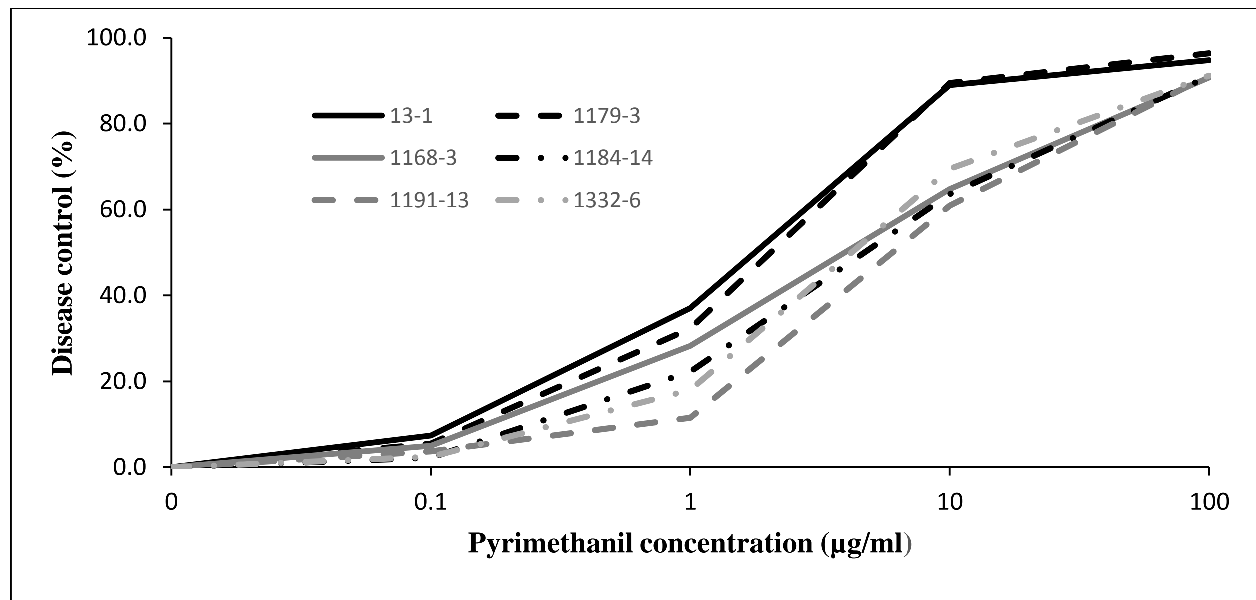

Figure 11. Mean *in vivo* percentage disease control of *Alternaria solani* isolates by pyrimethanil as determined in greenhouse assays.

DISCUSSION

The majority of commercially acceptable potato cultivars are susceptible to early blight and brown spot (Franc and Christ, 2001) and cultural practices are insufficient to reduce the inoculum, thus frequent applications of foliar fungicides are necessary for disease management. The incidence of fungicide resistance has grown substantially due to the introduction of fungicides with a single mode of action (Skylakakis, 1982). Due to countless examples of plant pathogens with reduced-sensitivity or resistance to pesticides, the plant protection community has taken steps to avoid or delay resistance development in newly developed chemistries including the formation of FRAC. One of the key elements in resistance management is the establishment of baseline sensitivity and the monitoring of pathogen populations over time. Establishment of baseline sensitivity of a pathogen to a fungicide is the first step needed to detect fungicide resistance (Jutsum et al., 1998; Russell, 2005). Further monitoring of a fungal plant pathogen can then detect shifts in pathogen sensitivity, predict efficacy of fungicide regimes, and recommend necessary resistance management tactics (Thomas et al., 2012). The primary objective of this study was to monitor the sensitivity of *A. solani* and *A. alternata* field populations to DMI and AP fungicides in order to determine if decreased sensitivity existed among those isolates.

A key element in a fungicide resistance monitoring program is to utilize an *in vitro* assay method that will most accurately measure the sensitivity of a particular pathogen to different modes of action. Therefore, an additional objective of this study was to compare two *in vitro* assay methods commonly used to determine the sensitivity of *Alternaria* spp. to SDHI fungicides. Results from this study indicated conidial germination and mycelial growth inhibition methods were significantly different when comparing EC_{50} values. The EC_{50} values of 57 *A.*

solani isolates obtained with the conidial germination method in this study were lower than those determined for the same isolates using the mycelial growth method, suggesting that conidial germination may be more sensitive than the mycelial growth in evaluating the inhibitory effects of SDHI fungicides against *A. solani*. However, the conidial germination inhibition generated significantly higher EC_{50} values than the mycelial growth inhibition for determining *A. alternata* sensitivity to fluopyram. This difference in assay methods was observed in another *in vitro* study that assessed sensitivity of *A. alternata* to boscalid (Avenot and Michailides, 2007). In this previous study, it was found that mycelial growth may be more sensitive than conidial germination, when SDHI fungicides act on *A. alternata*. Therefore, in future monitoring studies, the conidial germination method and mycelial growth method can be used to determine the sensitivity of *A. solani* and *A. alternata*, respectively, to SDHI chemistries.

This is the first report of monitoring sensitivity levels of *A. solani* and *A. alternata* populations to difenoconazole, metconazole, and pyrimethanil across multiple years and production areas. The two DMI fungicides were labeled recently for use on potato, but pyrimethanil has been used by growers for the management of early blight and brown spot for almost 10 years. The *in vitro* sensitivities of the fungicides described in this study are based on EC_{50} values derived from the inhibition of mycelial growth. Assays developed in other pathogen systems have successfully used this method (Bolton et al., 2012; Hilber and Schüepp, 1996). DMI fungicides and pyrimethanil inhibit mycelial growth of fungal pathogens, the development stage related to progression and proliferation of subcuticular stromata, but cause no inhibition of spore germination (Daniels and Lucas, 1995; Smith et al., 1991). It has been determined previously, that complex media such as malt-agar was not suitable for *in vitro* assays of AP fungicides, if mycelial plugs are used as inoculum from agar plates that have been incubated for

more than 23 h (Hilber and Schüepp, 1996). In that study, the incubation period of the inoculum did not affect the activity of anilino-pyrimidines, when L-asparagine (asp-agar) was used. Therefore, the synthetic medium containing asp-agar was used for *in vitro* pyrimethanil testing in this study.

Baseline isolates of *A. solani* and *A. alternata* used in this study had relatively high levels of variability in response to difenoconazole, with the difference between the most and least sensitive isolates being 12- and 11-fold, respectively. Similarly, wider distribution was reported in the sensitive *V. inaequalis* isolates, the causal agent of apple scab (Villani et al., 2015). However, the distribution ranges of difenoconazole sensitivity reported in *C. beticola* (Bolton et al., 2012), *C. coccodes* (Olaya et al., 2010), and *D. bryoniae* (Thomas et al., 2012) were narrow, displaying limited variation within baseline isolates. A few reports assessing *in vitro* fungicide sensitivity of metconazole on other fungal pathogens are available. The range of EC_{50} values for metconazole was narrow for baseline *A. solani* isolates (4.5-fold) when compared to the substantial variation reported in *Fusarium graminearum* (Spolti et al., 2014), and was similar to that reported in *Galactomyces geotrichum* (McKay et al., 2012). The range of sensitivity of *F. graminearum* isolates in response to metconazole was higher (17.2-fold) than what this study reports for *A. solani*; however, since those isolates had previous exposure to tebuconazole, they do not portray a valid baseline group for *F. graminearum*. In the baseline sensitivity established in the current study for *A. alternata*, there is a 12-fold difference in sensitivity from the most metconazole sensitive isolate to the least sensitive. The distribution of pyrimethanil sensitivity of baseline *A. solani* and *A. alternata* isolates in this study was narrow and is comparable with ranges of EC_{50} values reported in *B. cinerea* (Sun et al., 2010) and *V. inaequalis* (Köller et al., 2005), and unlike the wider distribution reported in *Penicillium* spp. (Sholberg et al., 2005).

The two DMI fungicides, difenoconazole and metconazole appear to exhibit great intrinsic activity against both *A. solani* and *A. alternata* due to their post-infection activity (Wong and Midland, 2007). The sensitivity of the majority of *A. solani* isolates collected from 2010 to 2014 was consistent with baseline isolates, therefore, these isolates remain sensitive to the two DMI chemistries. Even though a decrease in *in vitro* DMI sensitivity was observed in a small number of *A. solani* isolates, no loss of disease control has been reported when these chemistries are used in commercial fields. *In vivo* trials were not conducted assessing disease control provided by these fungicides, as previous studies have demonstrated that 2- to 4-fold changes in resistance factor (RF) values do not affect early blight disease control under greenhouse conditions (Pasche et al., 2004, 2005). It should be asserted, that DMIs are not the main 'specialty' fungicides used to combat early blight. Instead, tank mixtures of QoIs and SDHIs with protectant fungicides such as chlorothalonil and mancozeb are used in fungicide rotation programs, but these results do not necessarily indicate that the pathogen populations may not shift toward DMI resistance in the future.

The DMIs, a subclass of sterol biosynthesis inhibitors, inhibit the demethylation of lanosterol by the cytochrome P450 lanosterol 14α-demethylase gene (CYP51A1), as well as possibly the C-22 desaturase site (Brent and Holloman, 2007a; Wong and Midland, 2007). Despite their site-specific mode of action, DMI triazole fungicides are considered to be at medium risk because a multi-step process is involved in developing resistance (Brent and Holloman, 2007a; FRAC, 2015). DMI resistance development is quantitative, and fungi are thought to acquire several mutations over time to overcome the fungicide (Brent and Holloman, 2007b). This pattern contrasts with the qualitative change in populations observed for other site-specific fungicides of QoIs and SDHIs. In addition, QoI fungicides are rated as having a high

risk for development of fungicide insensitivity and a medium-to-high risk for SDHIs, possibly due to single-gene mutations conferring resistance (Brent and Holloman, 2007b; FRAC, 2015). The main mechanisms of DMI resistance reported in other pathogen systems are overexpression of the *CYP51A1* gene in *V. inaequalis* (Schnabel and Jones, 2007), point mutations in the *CYP51A1* gene in *Uncinula necator* (Delye et al., 1997), and overexpression of genes that are involved in ATP-binding cassette (ABC) transporter modulators in *M. graminicola* (Stergiopoulos and De Waard, 2002).

FRAC states that it is wise to accept that cross-resistance is present among DMI fungicides active against the same fungus. Examples of cross-resistance among the same chemical class have been reported in *Alternaria* spp. and other closely related fungi, so there is a high risk for cross-resistance between these two DMI fungicides in *A. solani* and *A. alternata* (Gudmestad et al., 2013; Pasche et al., 2004, 2005). However, given the fact that *A. solani* developed resistance against fungicides with single mode of action quite rapidly, it is highly likely that sequential use of these two DMIs in the same fungicide regime may increase the selection pressure on the pathogen population (Thomas et al., 2012). Currently, DMI fungicides are applied as mixtures to reduce the shift towards fungicide insensitivity. Difenoconazole is registered for use on potato in combination with mandipropamid as Revus Top™ (Syngenta Crop Protection) and metconazole as Quash™ (Valent U.S.A. Corporation). Difenoconazole has a limitation of no more than two consecutive applications before rotating to an alternate mode of action and metconazole has a restriction of two applications per season (Friskop et al., 2015). Increased risk of reduced-sensitivity towards difenoconazole, has been reported in *V. inaequalis* populations in Uruguay (Mondino et al., 2015). In that study, RF values of 6.6 and 11.7 were

reported in apple orchards with moderate (up to four applications per season) and intensive use (more than five applications per season) of the DMI chemistry, respectively.

Reduced-sensitivity of *A. solani* and *A. alternata* to pyrimethanil was first detected in field isolates collected in 2010 from Idaho (Fairchild et al., 2013). In that study, 4 out of 21 *A. solani* and 1 out of 9 *A. alternata* isolates were reported as resistant, although EC_{50} values were reported only for two isolates. The spiral gradient dilution method, an alternative to the classical poisoned agar plating technique was utilized to determine *in vitro* fungicide sensitivity of those isolates from Idaho. Despite the use of an alternative fungicide sensitivity screening method, PDA agar, a complex media was used for the *in vitro* assays. The use of complex media instead of synthetic media, might have had inhibitory effects on pyrimethanil, as a previous study has demonstrated that the activity of AP fungicides in fungicide sensitivity assays is low when complex media are used ((Hilber and Schüepp, 1996). The use of complex media, along with small sample size, might have led to the detection of relatively high frequency of resistance in the Idaho pathogen population. In the study reported here, 109 *A. alternata* isolates across numerous locations and years that were tested for sensitivity to pyrimethanil remain sensitive. In contrast, 6 out of 245 *A. solani* isolates exhibited reduced-sensitivity to the AP fungicide. These isolates demonstrated approximately 4- to 64-fold loss in sensitivity compared to the baseline population.

As expected, the level of disease control loss was unvarying with the EC_{50} values obtained from *in vitro* sensitivity assays. Pyrimethanil provided similar levels of control of early blight disease caused by sensitive *A. solani* isolates, which was significantly superior to the control provided on reduced-sensitive isolates. Reduced-sensitive isolates were not controlled by pyrimethanil except at the highest concentration. In this study, the term “reduced-sensitivity”

was used instead of resistance to describe the shift in sensitivity. Resistance to a specific fungicide should equate to a 100% loss of disease control, at every concentration tested demonstrating the fungicide would be of no value to the potato grower (Pasche et al., 2004). The *in vivo* data of this study do not portray a total loss of disease control, hence, the term “reduced-sensitivity” was used to reflect the shift in sensitivity of the *A. solani* population in response to pyrimethanil.

The single-site mode of action of AP fungicides has been suggested to result in the inhibition of the secretion of fungal hydrolytic enzymes essential during infection, as well as interfere with biosynthesis of methionine (Heye et al., 1994; Masner et al., 1994). Pyrimethanil, along with other AP fungicides cyprodinil and mepanipyrim, are considered to be medium risk chemistries in the development of fungicide resistance in fungal pathogens (FRAC, 2015). However, resistance to AP fungicides has been reported in *B. cinerea* (Amiri et al., 2013; Chapeland et al., 1999; Fernández-Ortuño et al., 2013; Hilber and Hilber-Bodmer, 1998; Latorre et al., 2002; Leroux et al., 1999; Moyano et al., 2004; Myresiotis et al., 2007; Sun et al., 2010), *Oculimacula* spp. (Leroux et al., 2013), *Penicillium digitatum* and *Penicillium expansum* of apple and citrus, respectively, (Kanetis et al., 2008; Xiao et al., 2011), and *V. inaequalis* of apple (FRAC, 2015).

B. cinerea, one of the most important phytopathogenic fungi, is considered to be a high risk pathogen for fungicide resistance development and resistance to AP fungicides is common in some field crops and greenhouses (Brent and Holloman, 2007b). Different phenotypes of AP resistance have been detected particularly in *B. cinerea*, with resistance levels varying from low to very high (Leroux et al., 1999; Myresiotis et al., 2007). Cross-resistance in *B. cinerea* has also been reported among the three fungicides within the AP class (Hilber and Schüepp, 1996;

Latorre et al., 2002; Myresiotis et al., 2007). *B. cinerea* has a high risk of AP resistance development due to resistant isolates demonstrating to be fit as sensitive isolates based on parameters of lesion growth and sporulation (Fernández-Ortuño et al., 2013; Moyano et al., 2004). Genetic information is a crucial element in determining resistance risk to AP fungicides in *B. cinerea* populations. Genetic analysis of *Botryotinia fuckeliana*, the teleomorph of *B. cinerea*, indicated that resistance to AP fungicides segregated in a 1:1 ratio, and therefore, the grey mold pathogen displays a high inherent resistance risk to this specific chemistry due to its monogenic resistance (Chapeland et al., 1999; Hilber and Hilber-Bodmer, 1998). It is believed that single-gene mode of resistance resulted in approximately 50% resistant isolates in some research studies focused on AP resistance in *B. cinerea* (Amiri et al., 2013; Fernández-Ortuño et al., 2013; Myresiotis et al., 2007). This major gene resistance reported in *B. cinerea* suggests that change in sensitivity should be rapid once resistant strains were detected. In contrast, field isolates *Oculimacula* spp. (formerly *Tapesia* spp.) displayed a gradual shift in sensitivity, suggesting polygenic control of resistance (Babij et al., 2000). This implies that there might be more than one mechanism of AP resistance in cereal eyespot fungi.

The research reported here lay the groundwork for monitoring the shift in sensitivity in *A. solani* and *A. alternata* to difenoconazole, metconazole, and pyrimethanil, thereby evaluating the efficacy of resistance management programs for these pathogens in intensive production systems. It is important to collect more pathogen isolates from potato production regions to monitor sensitivity to those fungicides. Monitoring studies will be valuable for continuous use of those chemistries in disease management programs. The two DMI fungicides and pyrimethanil should be used in rotation with other systemic and protectant fungicides to safeguard their efficacy. The risk of resistance to pyrimethanil developing in *A. solani* may be increased because

of preexisting resistance to QoIs and boscalid. The *A. solani* isolates that demonstrated reduced-sensitivity to pyrimethanil in this study contains mutations that confer resistance to strobilurins and boscalid (Mallik et al., 2014). The qualitative nature of resistance may be the reason for reduced-sensitivity towards pyrimethanil. Similar changes in population sensitivity have been observed for QoIs and SDHIs (Brent and Holloman, 2007b). Qualitative resistance is defined as a sudden and marked loss of efficacy and the presence of definite sensitive and resistant target population with extensively differing responses (Brent and Holloman, 2007a). The primary mode of action is yet undiscovered for AP chemistries, therefore, the resistance mechanism is currently unknown for the established fungicide group.

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APPENDIX A. COLLECTION DATA OF *ALTERNARIA SOLANI* ISOLATES COLLECTED FROM FIELDS IN THE 2010, 2011, 2012, 2013, AND 2014 SEASONS FROM SEVERAL STATES

Year	Isolate	Location	Cultivar
2010	1168 -1	Acequia, ID	Unknown
2010	1168 -3	Acequia, ID	Unknown
2010	1168 -7	Acequia, ID	Unknown
2010	1169 -4	Acequia, ID	Unknown
2010	1169 -7	Acequia, ID	Unknown
2010	1169 -10	Acequia, ID	Unknown
2010	1170 -1	Acequia, ID	Unknown
2010	1170 -4	Acequia, ID	Unknown
2010	1170 -7	Acequia, ID	Unknown
2010	1171 -4	Acequia, ID	Unknown
2010	1171 -7	Acequia, ID	Unknown
2010	1172 -6	Acequia, ID	Unknown
2010	1172 -8	Acequia, ID	Unknown
2010	1173 -6	Acequia, ID	Unknown
2010	1173 -7	Acequia, ID	Unknown
2010	1174 -1	Acequia, ID	Unknown
2010	1174 -5	Acequia, ID	Unknown
2010	1174 -9	Acequia, ID	Unknown
2010	1175 -2	Acequia, ID	Unknown
2010	1175 -4	Acequia, ID	Unknown
2010	1175 -8	Acequia, ID	Unknown
2010	1176 -3	Acequia, ID	Unknown
2010	1176 -5	Acequia, ID	Unknown
2010	1176 -7	Acequia, ID	Unknown
2010	1176 -8	Acequia, ID	Unknown
2010	1177-4	Oakes, ND	Unknown
2010	1177 -7	Oakes, ND	Unknown
2010	1178-E1	Acequia, ID	Unknown
2010	1178-W1	Acequia, ID	Unknown
2010	1179 -1	Pettibone, ND	Unknown
2010	1179 -2	Pettibone, ND	Unknown
2010	1179 -3	Pettibone, ND	Unknown
2010	1179-4	Pettibone, ND	Unknown
2010	1179 -5	Pettibone, ND	Unknown
2010	1179 -7	Pettibone, ND	Unknown

Year	Isolate	Location	Cultivar
2010	1179-8	Pettibone, ND	Unknown
2010	1179 -9	Pettibone, ND	Unknown
2010	1179 -10	Pettibone, ND	Unknown
2010	1179 -11	Pettibone, ND	Unknown
2010	1179-13	Pettibone, ND	Unknown
2010	1179-14	Pettibone, ND	Unknown
2010	1180-1	Pettibone, ND	Unknown
2010	1180-2	Pettibone, ND	Unknown
2010	1181 -1	O'Neil, NE	Unknown
2010	1181 -2	O'Neil, NE	Unknown
2010	1181 -3	O'Neil, NE	Unknown
2010	1181-5	O'Neil, NE	Unknown
2010	1181 -7	O'Neil, NE	Unknown
2010	1181 -8	O'Neil, NE	Unknown
2010	1181-9	O'Neil, NE	Unknown
2010	1181 -10	O'Neil, NE	Unknown
2010	1181 -12	O'Neil, NE	Unknown
2010	1181 -13	O'Neil, NE	Unknown
2010	1181 -14	O'Neil, NE	Unknown
2010	1181 -15	O'Neil, NE	Unknown
2011	1182-9	Columbus, NE	FL 1867
2011	1182-13	Columbus, NE	FL 1867
2011	1184-3	Wray, CO	FL 1867
2011	1184-14	Wray, CO	FL 1867
2011	1184-15	Wray, CO	FL 1867
2011	1184-20	Wray, CO	FL 1867
2011	1185-4	Bridgeport, NE	FL 1879
2011	1185-7	Bridgeport, NE	FL 1879
2011	1185-14	Bridgeport, NE	FL 1879
2011	1185-18	Bridgeport, NE	FL 1879
2011	1187-7	Columbus, NE	FL 1867
2011	1187-11	Columbus, NE	FL 1867
2011	1188-9	Columbus, NE	FL 1867
2011	1188-13	Columbus, NE	FL 1867
2011	1188-18	Columbus, NE	FL 1867
2011	1189-3	Columbus, NE	FL 1867
2011	1189-7	Columbus, NE	FL 1867
2011	1189-19	Columbus, NE	FL 1867
2011	1190-4	Wadena, MN	Unknown
2011	1190-7	Wadena, MN	Unknown
2011	1190-14	Wadena, MN	Unknown

Year	Isolate	Location	Cultivar
2011	1190-16	Wadena, MN	Unknown
2011	1191-2	Wadena, MN	Unknown
2011	1191-8	Wadena, MN	Unknown
2011	1191-13	Wadena, MN	Unknown
2011	1192-2	Wadena, MN	Unknown
2011	1192-7	Wadena, MN	Unknown
2011	1192-10	Wadena, MN	Unknown
2011	1192-15	Wadena, MN	Unknown
2011	1198-14	O'Neil, NE	Unknown
2011	1198-22	O'Neil, NE	Unknown
2011	1199-2	O'Neil, NE	Unknown
2011	1199-11	O'Neil, NE	Unknown
2011	1200-6	O'Neil, NE	Unknown
2011	1201-5	O'Neil, NE	Unknown
2011	1201-23	O'Neil, NE	Unknown
2011	1215-6	Columbus, NE	FL 1867
2011	1215-12	Columbus, NE	FL 1867
2011	1215-19	Columbus, NE	FL 1867
2011	1216-3	Columbus, NE	FL 1867
2011	1216-15	Columbus, NE	FL 1867
2011	1217-6	Columbus, NE	FL 1867
2011	1217-12	Columbus, NE	FL 1867
2011	1218-4	Newberry, MI	FL 2053
2011	1218-21	Newberry, MI	FL 2053
2011	1219-7	Park Rapids, MN	Unknown
2011	1219-16	Park Rapids, MN	Unknown
2011	1219-23	Park Rapids, MN	Unknown
2011	1220-4	Park Rapids, MN	Unknown
2011	1220-22	Park Rapids, MN	Unknown
2011	1221-3	Park Rapids, MN	Unknown
2011	1221-15	Park Rapids, MN	Unknown
2011	1222-4	Park Rapids, MN	Unknown
2011	1222-15	Park Rapids, MN	Unknown
2011	1223-4	Park Rapids, MN	Unknown
2011	1223-11	Park Rapids, MN	Unknown
2011	1223-17	Park Rapids, MN	Unknown
2011	1224-4	Columbus, NE	FL 1833
2011	1224-13	Columbus, NE	FL 1833
2011	1224-16	Columbus, NE	FL 1833
2011	1225-1	Dalhart, TX	FL 2048
2011	1225-12	Dalhart, TX	FL 2048

Year	Isolate	Location	Cultivar
2011	1226-1	Dalhart, TX	FL 1867
2011	1226-8	Dalhart, TX	FL 1867
2011	1226-12	Dalhart, TX	FL 1867
2011	1227-9	Dalhart, TX	FL 2053
2011	1227-12	Dalhart, TX	FL 2053
2011	1227-16	Dalhart, TX	FL 2053
2011	1229-2	Dalhart, TX	FL 1867
2011	1229-19	Dalhart, TX	FL 1867
2011	1230-1	Dalhart, TX	FL 1867
2011	1230-2	Dalhart, TX	FL 1867
2011	1230-4	Dalhart, TX	FL 1867
2011	1230-9	Dalhart, TX	FL 1867
2011	1230-15	Dalhart, TX	FL 1867
2011	1231-4	Lisbon, ND	Umatilla Russet
2011	1231-9	Lisbon, ND	Umatilla Russet
2011	1232-9	Becker, MN	Unknown
2011	1232-19	Becker, MN	Unknown
2011	1233-7	Menomonie, WI	Unknown
2011	1233-13	Menomonie, WI	Unknown
2011	1233-22	Menomonie, WI	Unknown
2011	1234-2	Browerville, MN	Unknown
2011	1234-11	Browerville, MN	Unknown
2011	1236-3	Parkers Prairie, MN	Unknown
2011	1236-8	Parkers Prairie, MN	Unknown
2011	1237-3	Dawson, ND	Unknown
2011	1237-17	Dawson, ND	Unknown
2011	1237-22	Dawson, ND	Unknown
2011	1238-3	Lisbon, ND	Unknown
2011	1238-17	Lisbon, ND	Unknown
2011	1239-5	Lisbon, ND	Unknown
2011	1239-13	Lisbon, ND	Unknown
2011	1239-20	Lisbon, ND	Unknown
2011	1246-14	Acequia, ID	Unknown
2011	1246-15	Acequia, ID	Unknown
2011	1247-21	Acequia, ID	Unknown
2011	1248-3	Acequia, ID	Unknown
2011	1248-12	Acequia, ID	Unknown
2011	1249-2	Acequia, ID	Unknown
2011	1250-1	Acequia, ID	Unknown
2011	1250-7	Acequia, ID	Unknown
2011	1250-13	Acequia, ID	Unknown

Year	Isolate	Location	Cultivar
2011	1251-5	Acequia, ID	Unknown
2011	1251-7	Acequia, ID	Unknown
2011	1252-4	Acequia, ID	Unknown
2011	1252-8	Acequia, ID	Unknown
2011	1254-5	Acequia, ID	Unknown
2011	1254-9	Acequia, ID	Unknown
2012	1273-1	Dalhart, TX	FL 1867
2012	1276-3	Dalhart, TX	FL 2137
2012	1277-2	Columbus, NE	Unknown
2012	1278-1	Hancock, WI	Unknown
2012	1279-2	Wray, CO	FL 1867
2012	1280-3	Bridgeport, NE	FL 2053
2012	1282-1	Bridgeport, NE	FL 1879
2012	1283-1	Rupert, ID	Unknown
2013	1288-1	Erie, IL	FL 1867
2013	1309-3	Bath, IL	Red Norland
2013	1313-1	Dawson, ND	Unknown
2013	1317-14	Columbus, NE	FL 1867
2013	1318-11	Farmington, NM	Unknown
2013	1319-6	Farmington, NM	Unknown
2013	1320-3	Farmington, NM	Unknown
2013	1320-9	Farmington, NM	Unknown
2013	1321-2	Plover, WI	Unknown
2013	1321-14	Plover, WI	Unknown
2013	1322-8	Plover, WI	Unknown
2013	1323-3	Plover, WI	Unknown
2013	1323-8	Plover, WI	Unknown
2013	1324-2	Wray, CO	FL 1867
2013	1325-3	Wray, CO	FL 1867
2013	1326-1	Wray, CO	FL 1867
2013	1327-5	Wray, CO	FL 1867
2013	1328-6	Wray, CO	FL 1867
2013	1329-4	Wray, CO	FL 1867
2013	1330-6	Wray, CO	Lamoka
2013	1331-3	Dalhart, TX	Russet Norkotah
2013	1332-6	Dalhart, TX	Russet Norkotah
2013	1333-10	Dalhart, TX	Russet Norkotah
2013	1334-8	Dalhart, TX	Russet Norkotah
2013	1335-4	Dalhart, TX	Russet Norkotah
2013	1335-14	Dalhart, TX	Russet Norkotah
2013	1339-5	Inkster, ND	Unknown

Year	Isolate	Location	Cultivar
2013	1340-9	Minden, NE	FL 1867
2013	1341-5	Columbus, NE	FL 1867
2013	1342-8	Columbus, NE	FL 1833
2013	1344-7	Karlsruhe, ND	Ranger Russet
2013	1345-2	Oakes, ND	Unknown
2013	1346-6	Oakes, ND	Unknown
2013	1348-4	Oakes, ND	Unknown
2013	1350-3	Lisbon, ND	Unknown
2013	1351-6	Oakes, ND	Unknown
2013	1352-3	Oakes, ND	Unknown
2013	1355-2	Oakes, ND	Unknown
2013	1356-3	Browerville, MN	Unknown
2013	1357-5	Browerville, MN	Unknown
2013	1358-1	Browerville, MN	Unknown
2013	1361-4	Perham, MN	Snowden
2013	1362-2	Perham, MN	Umatilla Russet
2013	1363-2	Columbus, NE	Unknown
2013	1365-1	Wray, CO	Unknown
2013	1367-3	Three Rivers, MI	FL 2137
2013	1367-14	Three Rivers, MI	FL 2137
2013	1369-10	Wray, CO	Unknown
2013	1377-1	Karlsruhe, ND	Unknown
2013	1380-3	Perham, MN	Umatilla Russet
2013	1389-1	Wadena, MN	Russet Burbank
2013	1390-7	Perham, MN	Russet Burbank
2013	1407-5	Connell, WA	Unknown
2013	1409-1	Jerome, ID	Unknown
2013	1411-3	Dalhart, TX	Snowden
2013	1412-5	Dalhart, TX	FL 1867
2013	1413-8	Dalhart, TX	Snowden
2013	1414-9	Dalhart, TX	Snowden
2014	1423-3	Rupert, ID	Unknown
2014	1426-4	Olton, TX	Unknown
2014	1446-6	Dalhart, TX	FL 2053
2014	1447-2	Dalhart, TX	Snowden
2014	1448-7	Dalhart, TX	FL 1867
2014	1449-5	Dalhart, TX	FL 2048
2014	1450-2	Dalhart, TX	FL 2053
2014	1451-3	Dalhart, TX	FL 2048
2014	1452-3	Dalhart, TX	FL 1867
2014	1458-1	Bridgeport, NE	FL 2137

Year	Isolate	Location	Cultivar
2014	1459-2	Alliance, NE	FL 2137
2014	1460-4	Columbus, NE	FL 1867
2014	1465-2	Karlsruhe, ND	Unknown
2014	1466-1	Karlsruhe, ND	Unknown
2014	1467-2	Three Rivers, MI	Silverton Russet

APPENDIX B. COLLECTION DATA OF *ALTERNARIA ALTERNATA* ISOLATES COLLECTED FROM FIELDS IN THE 2011, 2013, AND 2014 SEASONS FROM SEVERAL STATES

Year	Isolate	Location	Cultivar
2011	1183-4	O'Neil, NE	Unknown
2011	1186-1	Inkster, ND	Ranger Russet
2011	1186-5	Inkster, ND	Ranger Russet
2011	1202-6	Karlsruhe, ND	Ranger Russet
2011	1202-7	Karlsruhe, ND	Ranger Russet
2011	1202-8	Karlsruhe, ND	Ranger Russet
2011	1207-1	Inkster, ND	Unknown
2011	1209-2	Inkster, ND	Unknown
2011	1211-9	Inkster, ND	Unknown
2011	1211-11	Inkster, ND	Unknown
2011	1211-12	Inkster, ND	Unknown
2011	1211-14	Inkster, ND	Unknown
2011	1211-15	Inkster, ND	Unknown
2011	1213-20	Inkster, ND	Unknown
2011	1235-2	Parkers Prairie, MN	Unknown
2011	1235-3	Parkers Prairie, MN	Unknown
2011	1253-1	Acequia, ID	Unknown
2011	1255-1	Acequia, ID	Unknown
2011	1255-3	Acequia, ID	Unknown
2013	1285-1	Brawley, CA	Unknown
2013	1285-2	Brawley, CA	Unknown
2013	1285-3	Brawley, CA	Unknown
2013	1285-4	Brawley, CA	Unknown
2013	1285-8	Brawley, CA	Unknown
2013	1285-9	Brawley, CA	Unknown
2013	1286-1	Olton, TX	Unknown
2013	1286-2	Olton, TX	Unknown
2013	1286-3	Olton, TX	Unknown
2013	1287-1	Bath, IL	FL 1867
2013	1287-2	Bath, IL	FL 1867
2013	1287-3	Bath, IL	FL 1867
2013	1288-3	Erie, IL	Red Viking
2013	1288-5	Erie, IL	Red Viking
2013	1289-1	Cordova, IL	Goldrush
2013	1290-1	Savanna, IL	Superior

Year	Isolate	Location	Cultivar
2013	1290-2	Savanna, IL	Superior
2013	1291-1	Savanna, IL	Red Viking
2013	1292-1	Savanna, IL	Red Viking
2013	1292-2	Farmington, NM	Unknown
2013	1294-3	Farmington, NM	Unknown
2013	1295-2	Farmington, NM	Unknown
2013	1296-1	Farmington, NM	Unknown
2013	1296-2	Farmington, NM	Unknown
2013	1297-2	Farmington, NM	Unknown
2013	1298-1	Farmington, NM	Unknown
2013	1298-2	Farmington, NM	Unknown
2013	1299-1	Dalhart, TX	Russet Norkotah
2013	1299-2	Dalhart, TX	Russet Norkotah
2013	1300-1	Bath, IL	FL 1867
2013	1300-2	Bath, IL	FL 1868
2013	1301-2	Bath, IL	Atlantic
2013	1302-1	Colorado City, CO	Unknown
2013	1302-2	Colorado City, CO	Unknown
2013	1303-2	Lubbock, TX	Unknown
2013	1304-1	Colorado City, CO	Unknown
2013	1305-2	Colorado City, CO	Unknown
2013	1306-3	Erie, IL	Red Viking
2013	1306-4	Erie, IL	Red Viking
2013	1307-1	Savanna, IL	Superior
2013	1308-6	Cordova, IL	Gold Rush
2013	1308-7	Cordova, IL	Gold Rush
2013	1309-10	Bath, IL	Red Norland
2013	1309-11	Bath, IL	Red Norland
2013	1310-2	Cordova, IL	FL 1867
2013	1311-1	Olton, TX	Unknown
2013	1312-1	Dawson, ND	Unknown
2013	1312-2	Dawson, ND	Unknown
2013	1314-1	Dawson, ND	Unknown
2013	1315-1	Dawson, ND	Unknown
2013	1316-2	Columbus, NE	FL 1867
2013	1316-3	Columbus, NE	FL 1867
2013	1317-9	Columbus, NE	FL 1867
2013	1322-7	Plover, WI	Unknown
2013	1325-8	Wray, CO	FL 1867
2013	1327-8	Wray, CO	FL 1867
2013	1328-11	Wray, CO	FL 1867

Year	Isolate	Location	Cultivar
2013	1331-1	Dalhart, TX	Russet Norkotah
2013	1333-12	Dalhart, TX	Russet Norkotah
2013	1334-7	Dalhart, TX	Russet Norkotah
2013	1335-2	Dalhart, TX	Russet Norkotah
2013	1336-1	Cody, NE	Ranger Russet
2013	1337-2	Cody, NE	Ranger Russet
2013	1338-1	Cody, NE	Ranger Russet
2013	1338-2	Cody, NE	Ranger Russet
2013	1359-2	O'Neil, NE	Unknown
2013	1360-2	Perham, MN	Nicolet
2013	1366-2	Prosper, ND	Unknown
2013	1368-2	Minden, NE	Classic Russet
2013	1407-3	Connell, WA	Unknown
2013	1408-1	Kimberly, ID	Unknown
2013	1409-2	Jerome, ID	Unknown
2013	1409-3	Jerome, ID	Unknown
2013	1410-1	Kimberly, ID	Unknown
2013	1410-2	Kimberly, ID	Unknown
2014	1420-1	Dalhart, TX	Unknown
2014	1421-1	Dalhart, TX	Unknown
2014	1421-2	Dalhart, TX	Unknown
2014	1422-1	Acequia, ID	Unknown
2014	1422-2	Acequia, ID	Unknown
2014	1424-1	Olton, TX	FL 1867
2014	1425-1	Olton, TX	Unknown
2014	1425-2	Olton, TX	Unknown
2014	1426-1	Olton, TX	Unknown
2014	1426-2	Olton, TX	Unknown
2014	1427-1	Rupert, ID	Unknown
2014	1427-2	Rupert, ID	Unknown
2014	1453-3	Dalhart, TX	Lamoka
2014	1456-1	Dalhart, TX	FL 2048
2014	1456-2	Dalhart, TX	FL 2048

APPENDIX C. CV-8 AGAR MEDIUM

150 ml	Campbell's V-8 juice
1.5 gm	$CaCO_3$
15 gm	Agarose
900 ml	Distilled water

Mix 1.5 grams $CaCO_3$ with 150 ml V-8 juice, and clarify V-8 juice by spinning at 5,000 rpm for 5 min. Combine 100 ml of clarified supernatant, 900 ml ddH_2O, and 15 grams agarose; and autoclave at 15 psi for 20 min.

APPENDIX D. L-ASPARAGINE (ASP-AGAR) MEDIUM

1.31 gm	$K_2HPO_4.3H_2O$
1 gm	$MgSO_4.7H_2O$
0.5 gm	KCl
0.01 gm	$FeSO_4.7H_2O$
2 gm	L-asparagine
22 gm	$C_6H_{12}O_6.1H_2O$
	12M hydrochloric acid
15 gm	Agarose
990 ml	Distilled water

Five stock solutions were prepared for pyrimethanil sensitivity testing. Asp-agar consisting of $K_2HPO_4.3H_2O$ (1.31g) and $MgSO_4.7H_2O$ (1 g) each dissolved in 30 ml of distilled water, were stocks I and II, respectively. Stock III contained KCl (0.5g) and $FeSO_4.7H_2O$ (0.01g) dissolved 30 ml of distilled water. Stock IV was produced by dissolving L-asparagine (2 g) and agar (15 g) dissolved in 400 ml of distilled water. Stock V contains $C_6H_{12}O_6.1H_2O$ (22 g) dissolved in 490 ml of distilled water. The precipitate that forms when stocks I and II are combined was dissolved by adding 12M hydrochloric acid dropwise. A precipitate again was observed with the addition of stock III, but dissolved with the addition of stock IV. Stock solutions I-IV and stock V were combined after autoclaving and pyrimethanil was added after cooling.

APPENDIX E. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* COMPARISON OF CONIDIAL GERMINATION TO MYCELIAL GROWTH (FLUOPYRAM)

Table E1. Combined analysis of variance for *in vitro* sensitivity of *Alternaria alternata* to fluopyram based on conidial germination inhibition

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0688	0.63	0.4293
Isolate	49	1.7636	16.14	<0.0001
Experiment × Isolate	49	0.1256	1.15	0.2757
Error	100	0.1093		
Corrected Total	199			

Table E2. Combined analysis of variance for *in vitro* sensitivity of *Alternaria alternata* to fluopyram based on mycelial growth inhibition

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0592	0.49	0.4998
Isolate	49	3.6588	30.28	<0.0001
Experiment × Isolate	49	0.1464	1.21	0.2087
Error	100	0.1208		
Corrected Total	199			

Table E3. Summary of one-way analysis of variance comparing conidial germination inhibition and mycelial growth inhibition of *Alternaria alternata* in response to fluopyram

Source of Variation	Degrees of Freedom	Mean Square	F-value
Method	1	6.2500	9.2102*
Error	98	0.6786	
Corrected Total	99		

*= Significant difference ($P = 0.05$)

APPENDIX F. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* COMPARISON OF CONIDIAL GERMINATION TO MYCELIAL GROWTH (BOSCALID)

Table F1. Combined analysis of variance for *in vitro* sensitivity of *Alternaria solani* to boscalid based on conidial germination inhibition

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0172	0.69	0.2540
Isolate	56	0.3105	12.37	<0.0001
Experiment × Isolate	56	0.0193	0.77	0.0405
Error	114	0.0251		
Corrected Total	227			

Table F2. Combined analysis of variance for *in vitro* sensitivity of *Alternaria solani* to boscalid based on mycelial inhibition

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.1730	0.46	0.5003
Isolate	56	4.9317	13.03	<0.0001
Experiment × Isolate	56	0.6770	1.79	0.0046
Error	114	0.3784		
Corrected Total	227			

Table F3. Summary of one-way analysis of variance comparing conidial germination inhibition and mycelial growth inhibition of *Alternaria solani* in response to boscalid

Source of Variation	Degrees of Freedom	Mean Square	F-value
Method	1	200.14	304.1179*
Error	112	0.6581	
Corrected Total	113		

*= Significant difference ($P = 0.05$)

APPENDIX G. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* BASELINE SENSITIVITY TESTS OF *ALTERNARIA SOLANI* TO DMI FUNGICIDES

Table G1. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria solani* to difenoconazole

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0003	3.19	0.0769
Isolate	56	0.0117	108.48	<0.0001
Experiment × Isolate	56	0.0004	3.29	<0.0001
Error	114	0.0001		
Corrected Total	227			

Table G2. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria solani* to metconazole

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0000	0.03	0.8536
Isolate	56	0.0049	42.58	<0.0001
Experiment × Isolate	56	0.0003	2.26	0.0001
Error	114	0.0001		
Corrected Total	227			

APPENDIX H. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* BASELINE SENSITIVITY TESTS OF *ALTERNARIA ALTERNATA* TO DMI FUNGICIDES

Table H1. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria alternata* to difenoconazole

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0001	1.05	0.7095
Isolate	49	0.0216	167.50	<0.0001
Experiment × Isolate	49	0.0002	1.63	0.0206
Error	100	0.0001		
Corrected Total	199			

Table H2. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria alternata* to metconazole

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0002	2.01	0.1590
Isolate	49	0.0314	219.34	<0.0001
Experiment × Isolate	49	0.0002	1.52	0.0392
Error	100	0.0001		
Corrected Total	199			

APPENDIX I. SUMMARY OF STATISTICAL ANALYSIS FOR THE *IN VITRO* BASELINE SENSITIVITY TESTS OF PYRIMETHANIL

Table I1. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria solani* to pyrimethanil

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0001	0.22	0.6431
Isolate	56	0.0076	14.61	<0.0001
Experiment × Isolate	56	0.0009	1.77	0.0046
Error	114	0.0005		
Corrected Total	227			

Table I2. Combined analysis of variance for *in vitro* baseline sensitivity of *Alternaria alternata* to pyrimethanil

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	1	0.0001	0.35	0.8000
Isolate	49	0.0103	37.04	<0.0001
Experiment × Isolate	49	0.0004	1.59	0.0260
Error	100	0.0003		
Corrected Total	199			

APPENDIX J. SUMMARY OF STATISTICAL ANALYSIS FOR PYRIMETHANIL *IN VIVO* SENSITIVITY TESTS

Table J1. Combined analysis of variance of *in vivo* percentage disease control for sensitive and reduced-sensitive *Alternaria solani* isolates to pyrimethanil

Source of Variation	Degrees of Freedom	Mean Square	F-value	*P* value
Experiment	2	30.1031	3.08	0.0585
Rep	2	3.25480	0.33	0.7174
Isolate	5	1428.60	146.05	<0.0001
Rep × Isolate	10	5.57760	0.57	0.8368
Fungicide Concentration	3	90636.1	9265.94	<0.0001
Isolate × Fungicide Concentration	15	325.899	33.32	<0.0001
Error	178	9.78160		
Total	215			