2015-12-01

Control of *Alternaria solani* Resistance to Boscalid, Fluopyram, and Chlorothalonil

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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December 2015

ABSTRACT

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Alternaria solani, cause of early blight, threatens potato yields. Fungicide resistance has made control of early blight difficult and there are concerns that in-season fungicide use results in resistance to boscalid, fluopyram, and chlorothalonil. Concern of high levels of resistance to boscalid a group 7 fungicide may confer cross-resistance to fungicides of the same group such as fluopyram. From 2014 to 2015, *A. solani* isolates were collected from field plots treated with boscalid, fluopyram, and chlorothalonil to test resistance levels. Isolates were determined resistant if EC_{50} values were higher than 5 μg ml^{-1}. Boscalid and chlorothalonil mean EC_{50} values decreased two fold from 2014 to 2015, while fluopyram values increased two fold. A negative correlation between fluopyram and boscalid indicate no cross-resistance. Higher resistance levels to fluopyram (17.1 μg ml^{-1}) were observed in the treatment C-14 where only fluopyram was applied in 2014. Treatments D-14 and D-15, only treated with chlorothalonil, had the highest mean EC_{50} values to chlorothalonil (2.3 and 1.1 μg ml^{-1}, respectively). Field trials show fluopyram+chlorothalonil had lowest disease severity of 6.6 to 6.8%. Leaf residues of boscalid fluopyram, and chlorothalonil measured an average of 10.2, 4.9, and 55.0 ppm on leaves throughout the canopy. After 14 days average residues diminished to 0.74, 0.39, and 16.9 ppm for boscalid, fluopyram and chlorothalonil, respectively. Boscalid is not effective for early blight control because of high resistance; fluopyram resistance is increasing as treatments of fluopyram are applied; and chlorothalonil does not seem to be affected by continued fungicide application.

Keywords: cross-resistance, fitness, conidia, fungicide

ACKNOWLEDGEMENTS

I am very appreciative of the time and effort of those that have helped with this project. My committee has been a great help with helping me to develop the tools I would need to succeed. I owe much to my committee chair, Dr. Brad Geary who has put in so much time and patience as he consulted and reviewed the project and manuscripts. Dr. Jeff Miller has helped by allowing me to intern with Miller Research and has provided the resources of the study. Others at Miller Research LLC have helped in data collection. I express gratitude to Dr. Eggett of the Statistics Department for helping to understand how to use the tools of statistics in my study. I am also very thankful to the undergraduates who have helped me with the experiment preparations and collecting of data in the laboratory: thanks to Alex Dorrough, Zayne Callahan, and Daniel Reneer. I express appreciation for my wife and rest of my family for their constant support and encouragement.

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CHAPTER 1-Control of *Alternaria solani* Resistance to Boscalid, Fluopyram, and Chlorothalonil

INTRODUCTION

People of the United States consume potatoes (*Solanum tuberosum* L.) more than any other vegetable. The estimated value of potatoes in 2014 was $3.66 billion (USDA-NASS 2015). Diseases threaten to reduce potato yields up to 24% without the use of fungicides (Oerke 2006). Early blight, caused by *Alternaria solani* Sorauer, is a foliar disease of potatoes that affects most varieties grown throughout the world (Franc and Christ 2001). Early blight appears on the foliage as brown concentric rings with a yellow halo around the outside of lesions (Franc and Christ 2001). Infections can defoliate the crop, substantially reducing yields (Rotem 1994). Protectant fungicides used for early blight control can increase yields 18 to 39% (Harrison and Venette 1970; Horsfield et al. 2010). *Alternaria solani*, however, has a history of developing fungicide resistance. For example, *A. solani* populations developed resistance to quinone outside inhibitors (QoIs) two years after introduction (Pasche et al. 2004; Pasche et al. 2005). Current management strategies are four to six applications of fungicide per growing season in Idaho, where 30% of the potatoes in the U.S. are produced (USDA-NASS 2015). Other fungicides used for early blight control are mancozeb (manganese ethylenebis (dithiocarbamate) (polymeric)) and chlorothalonil (2, 4, 5, 6-tetrachlorobenzene-1, 3-dicarbonitrile), yet they are not as efficient in early blight protection as single-site inhibitors when under high disease pressure (Pasche and Gudmestad 2008; Stevenson and James 1999). Single-site inhibitors are frequently tank-mixed or alternated with multi-site inhibitors or other single-site inhibitors to expose *A. solani* to differing modes of action, but control continues to be a challenge for growers (Staub 1991).

Fungicides currently used to control early blight in Idaho include boscalid (2-chloro-N-[2-(4-chlorophenyl) phenyl] pyridine-3-carboxamide), fluopyram (N-{2-[3-chloro-5-(trifluoromethyl)-2-pyridyl] ethyl}-α, α, α-trifluoro-o-toluamide), and chlorothalonil. Boscalid (Endura; BASF Corporation, Agricultural Products, Research Triangle Park, NC) was registered for use in 2003, and is a carboxamide fungicide belonging to the succinate dehydrogenase inhibitors (SDHI), group 7 (FRAC 2015). SDHI fungicides affect cell respiration by targeting complex II of the mitochondrial respiratory chain and inhibiting the binding of ubiquinone to the docking-site between subunits B, C, and D of the succinate dehydrogenase enzyme (Avenot and Michailides 2010; Horsefield et al. 2006). Boscalid was initially effective in controlling *A. solani* when first registered (Pasche et al. 2005; Pasche and Gudmestad 2008). However, resistance to boscalid has been reported in *Alternaria spp.* isolates from potato and pistachio (Avenot and Michailides 2007; Gudmestad et al. 2013). In 2009, 15% of *A. solani* isolates collected from Idaho were described as resistant, while isolates sampled in 2010 reported resistance in 58% of the population (Fairchild et al. 2013). *In vitro* studies, such as Gudmestad et al.(2013) calculate resistance levels by finding the effective concentration that kills 50% of conidia (EC_{50}). Isolates collected from nine different states for this study observed 75% of *A. solani* isolates had *in vitro* EC_{50} values greater than 5 µg/ml, providing evidence of early blight resistance to boscalid spreading across potato-growing regions of the U.S. (Gudmestad et al. 2013). Resistant isolates of *A. solani* were found across the U.S. as early as 6 years following boscalid registration on potatoes.

Fluopyram (Luna Privilege, Bayer Crop Science, Durham, NC) was registered on potatoes in 2012 as Luna Tranquility (pre-mix of fluopyram and pyrmethanil). Fluopyram is also an SDHI (group 7) fungicide inhibiting complex II of the mitochondrial respiratory chain.

However, the chemical formula differs from that of boscalid, resulting in stronger binding within complex II of the mitochondrial respiratory chain (Fraaije et al. 2012; FRAC 2015). *Alternaria solani* isolates have not developed high levels of resistance to fluopyram (Fairchild et al. 2013; Gudmestad et al. 2013). In 2010, 4% of isolates tested in Idaho had reduced sensitivity, but it increased to 9% in 2011 (Fairchild et al. 2013; Miles et al. 2014). Since the emergence of boscalid resistance in *A. solani* isolates, some have postulated that *A. solani* might develop resistance to fluopyram given they are both in the same fungicide group 7. Cross-resistance occurs as pathogen populations develop resistance to one fungicide may also confer resistance to other fungicides of the same group such as carbendzim and thiabendazole of group 1 among *Botrytis cinerea* Pers. ex FR. isolates of grapevine (Leroux et al. 1999). However, cross-resistance is not observed to fluopyram and boscalid among *Alternaria spp.* (Avenot et al. 2014; Gudmestad et al. 2013). The lack of cross-resistance to fluopyram among *Alternaria spp.* resistant to boscaldid may be attributed to the different chemistries causing different binding affinities with boscalid and fluopyram (Fraaije et al. 2012; Scalliet et al. 2012). Mutations of the succinate dehydrogenase enzyme (complex II) decreases the binding affinity of boscalid to the ubiquinone docking-site (Scalliet et al. 2012). Fluopyram has not been observed to be affected by the mutations that cause resistance to boscalid in isolates of *Mycosphaerella graminicola* (Fückel) J. Schröt. In Cohn or *A. solani* (Mallik et al. 2014; Scalliet et al. 2012).

Chlorothalonil (Bravo, Syngenta Crop Protection, LLC, Greensboro, NC) is a multi-site inhibitor that reacts with glutathione and other thiols, disrupting enzyme activity in the cell (Tillman et al. 1973). Chlorothalonil, registered for use on potatoes in 1966, is often mixed with other fungicides such as boscalid and fluopyram and is considered to be at low risk for resistance development due to the multi-site activity (FRAC 2015). However, studies by Barak and

Edgington (1984) and Sujkowski et al. (1995) report low levels of resistance among *Phytophthora infestans* and *Botrytis cinerea* isolates. Fairchild et al. (2013) reported 35-40% of *A. solani* isolates collected from various sites in Idaho were resistant to chlorothalonil, which causes significant concern among growers because control options appear to be dwindling. Coupled with accounts of limited control under high disease pressure, there are concerns about chlorothalonil and SDHI fungicides becoming limited in their ability to control early blight (Gudmestad et al. 2013; Pasche and Gudmestad 2008).

Consistent exposure to fungicides via multiple applications during a growing season may drive a selection process, giving rise to fungicide resistance (Gudmestad et al. 2013; Rosenzweig et al. 2008). Resistant pathogen populations have a competitive fitness advantage as fungicides are used to control early blight. Fitness is defined as the survival and reproductive success of an allele, individual, or group and can be advantageous or a cost to the pathogen (Karaoglanidis et al. 2011). If resistance to boscalid, fluopyram, and cholorothalonil could be attributed to continued exposure of fungicides, the mutation may be a cost to the pathogen after the fungicide is no longer used as a selection factor. However, not all mutations result in a fitness cost to the resistant isolate; *Plasmopara viticola* isolates resistant to QoIs were observed to have higher infection frequencies than sensitive isolates (Corio-Costet et al. 2011). However, fitness costs have been seen in powdery mildew (*Erysiphe graminis* f.sp. *hordei*) of wheat as isolates resistant to quinoxyfen produced less spores (Hollomon et al. 1997). A fitness cost was also observed in the lower winter survival rates of the sclerotia of *B. cinerea* isolates resistant to iprodione (Raposo et al. 2000). The change of fungicide treatments on an annual basis may be enough to exploit less spores produced, slower growth rates, lower survival rates, etc. of fungicide-resistant isolates, particularly in boscalid, fluopyram and chlorothalonil. Therefore, the objectives of this

research are to (i) determine if populations of *A. solani* collected from areas where boscalid, fluopyram, and chlorothalonil were applied differ in their sensitivity to the respective fungicides; (ii) determine if boscalid resistant isolates are also resistant to fluopyram (cross-resistant); (iii) determine if there is a fitness cost for maintaining resistance to boscalid, fluopyram, and chlorothalonil.

MATERIALS AND METHODS

Field trial maintenance

Trials were established at Miller Research Experimental Farm near Acequia, ID. Certified disease-free potato seed (cv. Russet Burbank) used in trials were purchased from a commercial potato grower. Plots within each trial were 3.7 m. (four rows) wide and 8.8 m long with a 2.1 m border between plots. Treatments (2014: A-14, B-14, C-14, D-14, E-14; 2015: A-15, B-15, C-15, D-15; fungicides used in the treatments are listed in Table 1) were established according to a randomized complete block design with four replications (Table 1). Treatments were also chosen based on industry sponsors and fungicide use in Idaho. Treatment E-14 was not continued in 2015 based on industry protocol for trial.

Fungicide applications were made using the Miller Research ground plot sprayer (a small self-propelled tractor with a hydrostatic drive). The tractor carries twelve 11.4 liter capacity stainless steel tanks in which product was mixed and a Teflon-coated laboratory magnet was placed inside the tank to stir the mix as a second magnet located under the tank turned with a hydraulic motor; allowing for constant agitation of the spray mixture during application. Spray tanks were pressurized to 20 psi with compressed air and connected to the spray manifold with one-way valves to prevent an intermixing of spray solutions.

The spray boom consisted of eight TeeJet XR 11002 VS flat fan nozzles (Spraying Systems Co., Glendale Heights, IL) spaced 45.7 cm apart. Sprayer speed was measured at 6.0 k h^{-1} which resulted in a spray volume of 115.1 L ha^{-1}. The boom was positioned approximately 45.7 to 50.8 cm above the canopy. Treatment applications began just prior to row closure on June 21 and all treatments except D-14, were planned on a two-week schedule (Table 1) based on growers and industry requests of early blight trials in Idaho. Treatment D-14 received applications weekly for six total applications in 2014 however, the industry in Idaho requested a trial of only 4 applications in 2015.

Isolation of Alternaria solani

Potato leaves were collected in September 2, 2014 and August 12, 2015 from field trial plots. The field trials were established to test the efficacy of fungicides boscalid, fluopyram, and chlorothalonil to control *A. solani* (Table 1). Twenty to 25 leaf samples were collected in 2014 and eight to 11 samples in 2015 from each of the four blocks of treatments in Table 1. Leaf samples of differing blocks and treatments were kept separate. All leaf samples were taken to Brigham Young University in Provo, Utah, where they were pressed and dried in preparation for *A. solani* isolation.

Dried leaflets were sterilized in 10% sodium hypochlorite for 1-2 min. They were then washed in autoclaved distilled water, blotted dry on a paper towel, and then placed in 70% ethyl alcohol for 30 seconds. Leaves were then blotted dry and pieces were cut and placed on water agar. After four days, conidia were isolated using a glass needle and the aid of a microscope at 60x magnification. Conidia were transferred onto water agar, then separated for single spore isolation and transported to V8 media similar to Gudmestad et al. (2013). Media was modified slightly by not centrifuging the V8 juice before adding it to the media. Isolates were grown for

10-14 days under 24-hours of 40 watt fluorescent bulbs. Once mycelium covered the media, 4-mm plugs were placed in 2-ml centrifuge tubes, filled with 1 ml 15% glycerol solution, and stored in a freezer at -80° C. A total of ten isolates from each treatment (Table1), two to three isolates from each block, were randomly selected for *in vitro* assessment of fungicide sensitivity.

In vitro assessment of fungicide sensitivity

Thirteen to15-day-old *A. solani* cultures were used for inoculum production. Cultures were grown under 40 watt fluorescent bulbs for 11-13 days at 21°C, and then placed in a dark incubator for 2-3 days at 21°C for conidia production. Using sterile distilled water and a glass rod, conidia were washed clean of the V8 media. Conidia concentration was determined using a hemacytometer (Bright-Line, Hausser Scientific, Horsham, PA) and adjusted to 5 x 10^4 conidia ml^{-1}. An aliquot of 150 µl of conidia suspension was then added to the surface of fungicide amended media and spread with a sterile glass rod. Media contained 2% bacteriological grade agar (J637 Agar, AMRESCO LLC, Solon, OH) and was amended with technical grade boscalid (99% active ingredient [a.i.]; BASF Corporation, Research Triangle Park, NC), fluopyram (97.78% a.i.; Bayer CropScience, Durham, NC), and chlorothalonil (98% a.i.; Syngenta Crop Protection, LLC, Greensboro, NC). Isolates were tested on amended media at the concentrations: 0.0, 1.0, 10.0, 100.0, 500.0, and 1000.0 µg ml^{-1}, 0.0 0.1 1.0, 10.0, 100.0 µg ml^{-1}, and 0.0, 0.1, 1.0, 5.0, 10.0 µg ml^{-1} for boscalid, fluopyram, and chlorothalonil respectively. The technical grade fungicides were dissolved in acetone. Concentrations over 10 µg ml^{-1} required the use of formulated product of boscalid (tradename Endura) (70% a.i.; BASF) and fluopyram (tradename Luna Privilege) (43.5% a.i.; Bayer). Salicylhydroxamic acid (SHAM) stock solution was added to the amended media because past research has found that QoI and SDHI fungicides prevent the pathogen from developing resistance via an alternative pathway and gives a better assessment

fungicide sensitivity (Pasche et al. 2004). The stock solution contained 10,000 micrograms per milliliter of methanol. Final concentration of SHAM in amended fungicide media was 100 µg ml^{-1}. All media had a final concentration of 0.1% acetone and 1% methanol.

The plates were incubated in an Enviromental Growth Chamber (EGC, Chagrin, Ohio) at 26°C for 15 hours under continuous fluorescent bulbs of 54 watts. Petri plates were then examined at 80x magnification for conidia that had a germ tube or multiple germtubes as long as the conidia, and counted as viable germination. The experiment was performed twice each year, 2014 and 2015, with fungicide concentrations being replicated twice in each experiment.

Effective concentration of 50% (EC_{50}) was determined by finding the relative germination (RG) compared to the untreated control. Relative germination is,

$$\frac{germinated\ conidia\ on\ fungicide\ amended\ media}{germinated\ conidia\ on\ non\ fungicide\ amended\ media} * 100$$

Binomial regression was used after using the ln(x+1) transformation of the concentration to predict EC_{50} concentrations. Isolates with estimated EC_{50} values to boscalid exceeding 1000 µg ml^{-1} were adjusted to 1000 µg ml^{-1} because extrapolated values could not be trusted and fell outside the range of tested concentrations. Previous studies determined that EC_{50} values above 5 µg ml^{-1} were considered resistant by comparing isolate EC_{50} values that have been exposed to the fungicide with baseline isolate *in vitro* EC_{50} values (Gudmestad et al. 2013; Pasche et al. 2004).

Leaf Residue analysis

In-field leaf residues were tested to determine the deposition of fungicides within a potato canopy immediately after fungicide application, and then again 14 days later. Ten leaflets were collected on June 23 and July 6 from the upper, middle, and lower canopy of the four blocks of treatments B-15, C-15, and D-15 (Table 1). A total of 120 leaflets were collected from each of the three treatments. Leaflets were then sent to OMIC analytical laboratories (OMIC USA Inc.,

Portland, OR) to obtain residue concentrations of boscalid, fluopyram, and chlorothalonil from the respective treatments: B-15, C-15, and D-15 at the three different levels of the canopy. Residues were obtained to help determine the potential exposure levels of *A. solani* isolates to boscalid, fluopyram, and chlorothalonil.

Isolate fitness

Fitness of resistant and non-resistant *A. solani* isolates were tested by mycelium growth over a seven day period, and by the number of spores produced after those seven days. Mycelial growth was measured by taking three 4-mm plugs from the 21-day-old cultures and placed each in the center of Petri dishes containing V8 agar. Two radial-growth measurements at 2, 4, 6, and 7 days were taken on three plates stored at 22°C under continuous 40 watt fluorescent light for four days. On the fifth day, lights were turned off for *in vitro* conidial production (Lukens 1960). After 7 days the conidia were washed with 5 ml of dH_2O and were counted using a hemacytometer. Both the mycelial growth and conidial production experiments were replicated twice.

Statistical Analysis

Natural log transformed mean (Ln) EC_{50} values of isolates to fluopyram and chlorothalonil were analyzed using two-way analysis of variance (ANOVA). Means were separated using Fisher's protected LSD test ($\alpha < 0.5$) when the treatments were significant. Boscalid EC_{50} values were analyzed using a Kruskal-Wallis comparison of ranked-sums test for non-parametric data. Spearman's ranked correlation coefficients between boscalid and fluopyram were determined to compare isolate EC_{50} values ($\alpha < 0.5$). Fitness data were analyzed by a linear model for mycelium growth and a two-sample t-test for spore production also using the Ln transformation. All analyses were done using R (R Foundation for Statistical Computing, 2013).

Early blight severity data were analyzed by ANOVA and means were separated using Fisher's protected LSD when the treatment effect was significant using Agricultural Research Manager (ARM) version 9 (Gylling Data Management, Brookings, South Dakota).

RESULTS

In vitro fungicide sensitivity

Estimated EC_{50} values of *A. solani* isolates varied among the different fungicide treatments and years (Figure 1). Boscalid EC_{50} values of *A. solani* isolates exhibited a wider range (17.1 to >1000 μg ml^{-1}) than what was observed with isolates of other studies. The EC_{50} values of fluopyram and chlorothalonil were not as variable (Figure 1). In 2014, boscalid values ranged from 128 to 1000 μg ml^{-1} with a mean of 853.5 μg ml^{-1}. Boscalid values decreased in 2015 by 2.2-fold with a mean EC_{50} value of 392.4 and ranged from 17.1 to 1000 μg ml^{-1} ($P < 0.001$) (Figure 1). The range of resistance found among isolates in 2015 indicated a drop in the level of resistance among *A. solani* isolates. Fluopyram EC_{50} values increased 2.04 fold between 2014 and 2015 ($P < 0.001$) with a range of 0.38 to 33.1 μg ml^{-1} and 2.71 to 58.5 μg ml^{-1}, respectively (Figure 1). The range of fungicide resistance changed little between years, but the number of resistant isolates increased from 60% in 2014 to 85% in 2015. Chlorothalonil values were significantly different ($P < 0.001$) between 2014 and 2015 decreasing 2.12-fold with ranges of 1.00 to 4.00 μg ml^{-1} and 0.24 to 1.97 μg ml^{-1}, respectively ($P < 0.001$) (Figure 1). As found for boscalid, the values for chlorothalonil decreased from 2014 to 2015, however, resistance was not observed. All isolates had EC_{50} values less than 5 μg ml^{-1}.

The boscalid EC_{50} values observed for all isolates collected in 2014 and 2015 were well above 5 μg ml^{-1} (Figure 1). However, there was not a significant difference among treatments in 2014 ($P = 0.34$) or 2015 ($P = 0.70$) (Table 2). Treatment B-14 had the highest mean EC_{50} value

to boscalid (1000 μg ml^{-1}). However, differences among treatments were not seen in sensitivity trials. Treatment B-15 did not yield isolates with the highest mean EC_{50} value in 2015 as treatment B-14 (Table 2).

Fluopyram-resistant isolates, having EC_{50} values greater than 5 μg ml^{-1}, were 60% in 2014 and 85% in 2015 (Figure 1). Fungicide treatments were significantly ($P < 0.001$) different, and the highest mean value of isolates was C-14 in 2014 with 17.06 μg ml^{-1} (Table 2). However, treatment C-15 in 2015, had a lower mean of 11.83 μg ml^{-1} and was not the most resistant. None of the treatments in 2015 were significantly different ($P=0.45$, Table 2). The lack of treatment effect may be a result of isolates with different sensitivities to fluopyram being collected (Table 2).

None of the isolates collected in 2014 and 2015 were resistant to chlorothalonil (Figure 1). There was a significant ($P = 0.002$) difference with treatment D-14 to B-14 and C-14 to chlorothalonil in 2014 but, no differences in 2015 (Table 2). Treatment D-14 had the highest EC_{50} mean of 2.29 μg ml^{-1}, and was again the highest in 2015 with a mean of 1.09 μg ml^{-1} (Table 2). The use of chlorothalonil without another fungicide was observed to have higher means. However, levels of treatments were not significant in 2015 with a range between treatments of 0.29 and 0.12 μg ml^{-1} ($P = 0.44$, Table 2).

Although a high percentage of *A. solani* isolates were observed to be resistant to boscalid, cross-resistance was not observed as isolates were considerably less resistant to fluopyram (Figure 1). Resistance observed to boscalid had no correlation with fluopyram resistance in 2014 ($r = -0.342$, $P = 0.015$) or 2015 ($r = -0.467$, $P = 0.002$) (Figure 2). The negative correlations found in both years suggests that there was no cross-resistance occurring within the *A. solani* isolates located within the plots.

Early blight severity

Severity of early blight in field plots were significant in both years ($P < 0.001$) (Table 2). In 2014 and 2015, treatments A-14 and A-15 had the highest mean percent severity at 34.7% and 36%, respectively. Treatments C-14 and C-15 had the lowest percent severity of 6.6% and 6.8%, respectively, and were significantly less than other treatments. While treatments B-14, D-14, and E-14 had significantly lower early blight severity than A-14, they were significantly higher than C-14 in 2014. In 2015, treatments B-15 and D-15 were not significantly different from each other but were significantly different than C-15. Treatments C-14 and C-15 are the only two treatments with fluopyram in the field trials (Table 1). Boscalid did not decrease disease severity when tank-mixed with chlorothalonil or other fungicides from treatment E-14 (Table 2).

Leaf residue of fungicides

The amount of boscalid, fluopyram, and chlorothalonil diminished down through the canopy, and after 14 days the amount decreased substantially (Figure 4). Boscalid and fluopyram had lower amounts of residue found on leaves than chlorothalonil. Rates of application were also lower for boscalid (281.3 ml ha^{-1}) and fluopyram (92.8 ml ha^{-1}) than chlorothalonil (947.1 ml ha^{-1}, Table 1). Boscalid and fluopyram residues after 14 days ranged from 0.37 to 0.96 ppm and 0.29 to 0.45 ppm, respectively. The amount of chlorothalonil residue substantially decreased from 1 day after the application of chlorothalonil to 1 days later on the upper canopy; however, the amount on the mid canopy diminished slightly in the time period signifying less degradation, while the residue on the lower canopy increased (Figure 4). The amounts of chlorothalonil residue on the mid and lower canopy were 22 and 24 ppm respectively. The amount of residue was well above the designated resistance level of 5 $\mu g\ ml^{-1}$.

Resistant vs sensitive isolate fitness

The growth rate of resistant *A. solani* isolates were not significantly different from non-resistant isolates (P = 0.24) (Figure 3-B). Resistant and non-resistant isolates grew similarly regardless of the year they were collected. The number of conidia mm^{-2} between resistant and non-resistant isolates showed sensitive isolates produced 1.32 more conidia mm^{-2} than resistant isolates (p =0 .052) (Figure 3-A). The slight increase of spore production may be a source of fitness advantage for sensitive isolates.

DISCUSSION

All isolates collected in this study were resistant to boscalid at rates far beyond the 5 ug ml^{-1} resistance threshold. The mean EC_{50} values in this study were 2000 fold greater than the baseline mean EC_{50} values in Gudmestad et al (2013).Based on the extensive use of boscalid from the time it was registered for use in 2005, the buildup of fungicide-resistant populations should not be surprising based on a study where Gudmestad et al. (2013) observed a 75% of isolates with boscalid EC_{50} values ranging from 15 to greater than 100 fold greater than EC_{50} values of baseline isolates. The boscalid EC_{50} isolates surveyed in 2010 and 2011 ranged from 0.5 to greater than 500 µg ml^{-1} with 75% of all isolates surveyed in 2010 and 2011 producing values greater than 5 ug ml^{-1} and in Idaho 42% of isolates returning values greater than 40 µg ml^{-1} (Gudmestad et al. 2013). Even with high levels of resistance to boscalid among isolates of *A. solani*, the mean EC_{50} value of 2015 was 2.18 fold less than in 2014. The decrease of EC_{50} values may be a result of less boscalid used in 2015 than in 2014 resulting in less-resistant isolates collected in 2015 than in 2014. If less boscalid were used year to year, non-resistant isolates may repopulate the area allowing boscalid to be more efficient in disease control. A similar trend was observed among isolates of *Sphaerotheca fuliginea*, powdery mildew on cucurbits (Schroeder

and Provvidenti 1969). *S. fuliginea* had been resistant to benomyl, however after 20 years of not using benomyl few isolates were observed to be resistant (McGrath et al. 1996).

Over the past few years, fluopyram has been highly efficient in early blight control in Idaho (Miller 2012) (Table 2). The increased use of products with fluopyram as an activie ingredient has been slow by growers due to the high costs and delaying the amount of resistance found among the *A. solani* population. Fairchild et al. (2013) reported about 4% of *A. solani* isolates were resistant to flupyram in 2010 and Miles et al. (2014) reported 9% of isolates were observed to be resistant to fluopyram in 2011; yet 73% of the isolates in this study were resistant to fluopyram (Figure 1). Values observed for fluopyram in our study were more than 42-fold higher than the baseline isolates in the Gudmestad et al. (2013) study. This high level of resistance may be attributed to the lack of genetic variability associated with the small area of the trial (mean 0.58 ha) or the amount of incubation time before collection of data. Treatments in 2014 were significantly different when tested against fluopyram during *in vitro* tests supporting the hypothesis that treatments where fluopyram were applied affected levels of resistance. However, the mean values of the treatments in 2015 were not significantly different from each other (Table 2). Treatment C-14 received two treatments of fluopyram and pyrimethanil without being mixed with chlorothalonil (Table 1). Treatment C-15 received two applications of fluopyram, pyrimethanil, and chlorothalonil, the tank-mix of chlorothalonil appears to have lowered the mean EC_{50} in 2015 and coincides with management strategies of using tank-mixes. However, as fluopyram is used with more frequency resistance levels are likely to increase as indicated with the increase in resistant isolates from 2014 to 2015 (Figure 1).

Cross resistance from boscalid is not influencing the development of *A. solani* resistant isolates to fluopyram. In fact, the correlation is negative, meaning cross-resistance is not

developing between these fungicides even though they are in group 7 (FRAC 2015) (Figure 2). This is supported in other papers dealing with the resistance of SDHI fungicides (Amiri et al. 2014; Gudmestad et al. 2013). Research on the binding affinity of fluopyram to the quinone binding site shows that fluopyram binds tighter than does boscalid due to the binding of ligands with the toxins and it decreases the ability of *A. solani* to develop resistance (Fraaije et al. 2012; Scalliet et al. 2012).

Chlorothalonil results show that D-14 and D-15 had the highest mean EC_{50} values, though none were resistant. These two treatments differed in applications between 2014 and 2015 (Table 2). Treatment D-14 received six applications of chlorothalonil while D-15 only received four applications, however there were no differences in disease severity between the two years (Table 1, Table 2). In a study done by Holm et al. (2003), field populations of *A. solani* were observed to be more resistant to chlorothalonil as the growing season progressed. Holm et al. (2003) also observed an effect in rate and number of applictions of chlorothalonil causing different mean EC_{50} values among fields. Our study observed higher EC_{50} values with more applications of chlorothalonil in treatment D-14 of 2014, EC_{50} values may be higher among isolates compared to treatment D-15 in 2015. Chlorothalonil is considered low risk for resistance development (FRAC 2015); however, sensitivities of isolates to chlorothalonil may vary according to how much is applied without tank-mixing products with different modes of action. Our results are different from those observed by Fairchild et al. (2013) who reported 35-40% resistance among *A. solani* isolates collected in Idaho. The methods of Fairchild et al. (2013) and our study differed in the use of light. Fairchild et al. (Fairchild et al. 2013) did not expose amended fungicide plates, with inoculum, to light during the incubation period, while we exposed our plates to light for 15 hours. Light is reported to be important in the activation of

chlorothalonil and influences the efficacy to *A. solani* conidia (Khan and Akhtar 1983; Peñuela and Barceló 1998). However, our study is not a representative selection of isolates from Idaho and inferences cannot be made about the population of *A. solani* across Idaho because our samples came from plots located in a single field. Chlorothalonil also saw a decrease in EC_{50} values from 2014 to 2015 (Figure 1). The decrease in EC_{50} values may be related to different field locations in 2014 and 2015 due to crop rotation. The different locations would likely have a different subset of the population.

Our study with all isolates were resistant to boscalid and 73% of all isolates were resistant to fluopyram (Figure 1). These results of boscalid and fluopyram are higher than previously reported (Gudmestad et al. 2013; Pasche et al. 2005). Pasche et al. (2005) and Gudmestad et al. (2013) evaluated conidia germination for the *in vitro* sensitivity tests after four hours of incubation which may result in lower percentage of germination and lower EC_{50} values. Tymon and Johnson (2014) incubated isolates for 24 hours giving conidia more time to germinate. We evaluated germination after 15 hours because conidia in control plates had nearly 100% germination at that time, which was considerably higher than germination after four hours incubation. We observed germination of conidia after 4 hours at high concentrations of boscalid but after 15 hours germination was much higher. We believe germination is delayed at high concentrations of boscalid but not killed. Extending the incubation period may have allowed relatively more spores to germinate, which would have likely been considered dead if evaluated after a 4-hour incubation period (Majchrowicz and Poprawski 1993). A more prolonged incubation period may have also given conidia more time to overcome any delayed germination responses.

As the amount of boscalid, fluopyram, and chlorothalonil application increases or decreases in the field, *A. solani* resistance seems to increase or decrease with the amount of respective fungicides used. Isolates collected from treatments in 2014 and 2015 did not yield different mean EC_{50} values to boscolid (Table 2). However, isolates among treatments did yield different mean EC_{50} values to fluopyram and chlorothalonil in 2014, but not in 2015 (Table 2). The differences observed per year may be in part due to the land area of this study causing plot to plot interference or contamination. To better test the effect of fungicide applications on levels of resistance in *A. solani*, it may be better to inoculate with one isolate of known EC_{50} value to boscalid, fluopyaram, and chlorothalonil than to rely on natural inoculum.

Early blight severity ratings from the field plots were significantly different and demonstrate the usefulness of fungicides, but they also show that EC_{50} values obtained from lab experiments do not always correspond directly with the field tests of disease severity according to the amount of resistance found among isolates to boscalid and the low amounts of disease severity (Table 2). It is unclear if boscalid was effective in the fungicide programs as B-14 and B-15 were not significantly different to D-14 or D-15. As long as tank-mixing fungicides with different modes of action continues to be a standard management practice, early blight severity will be less than non-treated plants (table 2). Due to the high levels of resistance to boscalid found in our *in vitro* studies, the use of boscalid alone is not recommended for early blight control, however boscalid continues to be effective against *Sclerotinia sclerotiorum* (Lib.) de Bary (white mold) control and so growers continue to mix it in their sprays (Kirk et al. 2009).

Based on our results fungicide-resistance levels observed in lab-based studies do not correlate to the concentrations of fungicides found in the field (Figure 4). Resistance in this study, and other previous studies, identified resistant isolates when germination occurred after

exposure to 5 or more µg ml^{-1} of fungicide (Gudmestad et al. 2013; Kim and Xiao 2010; Yin et al. 2011). Field residues are subjected to a number of variables, such as irrigation, photodegradation, and new plant growth, which diminish fungicide concentrations within the upper and middle canopies (Geary et al. 1999). The observed decreasing trend in residues of boscalid, fluopyram, and chlorothalonil followed similar trends observed by Geary et al. (1999) where fungicide concentrations were higher in all canopy levels immediately after application but decreased in the upper to mid-levels of the canopy prior to the next application. They also observed that as residues diminished, percent disease severity also increased. The loss of effective fungicide concentrations between two applications may be a factor in the development of resistance given that not all isolates are exposed to lethal rates of fungicide (Figure 3). If resistance is to be minimized, then an understanding of fungicide persistence within a canopy is necessary to maintain lethal concentrations. Fungicide residue analysis within this study was not comprehensive due to expensive laboratory procedures, but provided an overview of what was occurring within the field at the time of application and again 14 days later. Fungicide deposition trends in our study followed that of Geary et al., (1999). However, boscalid and fluopyram have not been tested as extensively as chlorothalonil.

Isolate fitness can be influenced by several different stages of pathogen life cycles. Mutations causing resistance can affect fitness throughout stages of the life cycle (Chapara et al. 2011; Karaoglanidis et al. 2011; Ma and Uddin 2009). Evidenced by enhanced fitness via increased spore production in wild-type *A. solani* isolates compared to QoI fungicide resistant isolates (Pasche and Gudmestad 2008). In our study, differences in mycelium growth were not significant. However, there was evidence that isolates not resistant to fluopyram produced more spores than resistant isolates and may help to control the frequency of isolates resistant to

fluopyram in the population (Figure 3, P = 0.052). In order to exploit this cost of fitness to fluopyram, one viable resistance management strategy would be to not use fluopyram every year in order for the sensitive isolates to predominate.

CONCLUSIONS

Field treatments of fluopyram and chlorothalonil affected the sensitivity levels of *A. solani*, which gives limited support that treatments alter sensitivity levels. In contrast, boscalid did not give any evidence of the effect of treatment on levels of resistance. However, resistance levels to boscalid were observed to decrease as growers in the area used less boscalid. Fluopyram resistance was observed among the small population sampled from the limited geographical region in the two years of our study. Non-resistant isolates produce more spores than do resistant isolates to floupyram, therefore management practices should not use fluopyram as often to select for non-resistant isolates. As is consistent with previous trials, resistance to boscalid does not confer resistance to fluopyram (Gudmestad et al. 2013). Better management practices can be determined from the results. Boscalid is not an effective fungicide in the control of early blight disease. Evidence of fluopyram resistance increases as fluopyram is used. Isolates are not resistant to chlorothalonil continues to control early blight and should be used with other fungicides in tank-mixes.

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TABLES AND FIGURES

Table 1. Fungicide treatments for control of early blight used in field at Miller Research in 2014 and 215.

Treatment	Fungicides	Rate[1]	no. Applications	Date of Application	Date of Application	Sample	% a.i[2]
2014							
A-14	Untreated Control	NA	NA	21-Jun	8-Jul	2-Sep	NA
B-14	Boscalid+Chlorothalonil	281.3 ml ha^{-1}, 631.4 ml ha^{-1}	2	22-Jul	6-Aug	2-Sep	70, 54
	Chlorothalonil	947.1 ml ha^{-1}	2	21-Jun	8-Jul		54
C-14	Fluopyram+Pyrimethanil	92.8 ml ha^{-1}, 277.0 ml ha^{-1}	2	22-Jul	6-Aug	2-Sep	11.3,33.8
	Chlorothalonil	947.1 ml ha^{-1}	2	21-Jun	8-Jul	2-Sep	54
D-14	Chlorothalonil	947.1 ml ha^{-1}	6	22-Jul	6-Aug	2-Sep	54
E-14	Boscolid+Mandipropamid+ Difenoconazole	281.3 ml ha^{-1}, 87.7 ml ha^{-1}, 87.7 ml ha^{-1}	2	21-Jun	8-Jul	2-Sep	70,21.9,21.9
	Pyrimethanil+Chlorothalonil	279.2 ml ha^{-1}, 631.4 ml ha^{-1}	2	22-Jul	6-Aug	2-Sep	54.6,54
2015							
A-15	Untreated Control	NA	NA	22-Jun	6-Jul	12-Aug	NA
B-15	Boscalid+Chlorothalonil	281.3 ml ha^{-1}, 631.4 ml ha^{-1}	2	22-Jul	4-Aug	12-Aug	70, 54
	Chlorothalonil	947.1 ml ha^{-1}	2	22-Jun	6-Jul		54
C-15	Fluopyram+Pyrimethanil+ Chlorothalonil	92.8 ml ha^{-1}, 277.0 ml ha^{-1}, 947.1 ml ha^{-1}	2	22-Jul	4-Aug	12-Aug	11.3, 33.8, 54
	Chlorothalonil	947.1 ml ha^{-1}	2	22-Jun	6-Jul	12-Aug	54
D-15	Chlorothalonil	947.1 ml ha^{-1}	4	22-Jul	4-Aug	12-Aug	54

[1], Rates were taken from the rate of formulated product multiplied by percent active ingredient.
[2], Percent active ingredient in formulated product.

Table 2. Results of *in vitro* EC_{50} values[1] (µg ml-1) and disease severity. Treatments are as follow: A-14 = untreated control, B-14 = boscalid+chlorothalonil, C-14 = fluopyram+ pyrimethanil +chlorothalonil, D-14 = chlorothalonil, E-14 = boscalid+mandipropamid+difenoconazole+pyrimethanil+chlorothalonil.

	Boscalid		Fluopyram		Chlorothalonil		Disease Severity
Year, Treatment	mean	range	mean	range	mean	range	%Area
2014							
A-14	956.9	643.7 to 1000	5.6 bc	1.9 to 24.1	2.1 ab	1.7 to 2.8	34.7 a
B-14	1000.0	NA*	2.6 c	0.4 to 8.5	1.8 bc	1.2 to 2.6	17.7 b
C-14	741.4	128.0 to 1000	17.1 a	6.9 to 33.1	1.5 c	1.00 to 3.2	6.6 c
D-14	795.6	178.7 to 1000	6.0 b	1.5 to 24.8	2.3 a	1.4 to 3.0	14.3 b
E-14	802.2	128.1 to 1000	6.5 bc	1.7 to 25.0	2.2 ab	1.8 to 4.0	16.9 b
p-vlaue (α=0.05)	0.3		< 0.001		0.002		< 0.001
2015							
A-15	302.9	17.1 to 1000	11.0	2.7 to 46.8	0.9	0.2 to 2.0	36.0 a
B-15	357.0	27.3 to 1000	11.0	4.3 to 36.5	1.0	0.5 to 1.8	14.1 b
C-15	410.4	89.0 to 1000	11.8	5.8 to 31.1	0.8	0.7 to 1.0	6.8 c
D-15	534.1	49.4 to 1000	18.3	5.4 to 58.5	1.1	0.7 to 1.8	16.1 b
p-vlaue (α=0.05)	0.7		0.452		0.443		< 0.001

*. All mean EC50 values were adjusted to 1000 µg ml-1 and therefore there is no range or SD.
[1]. Values are back transformed after ln transformation.

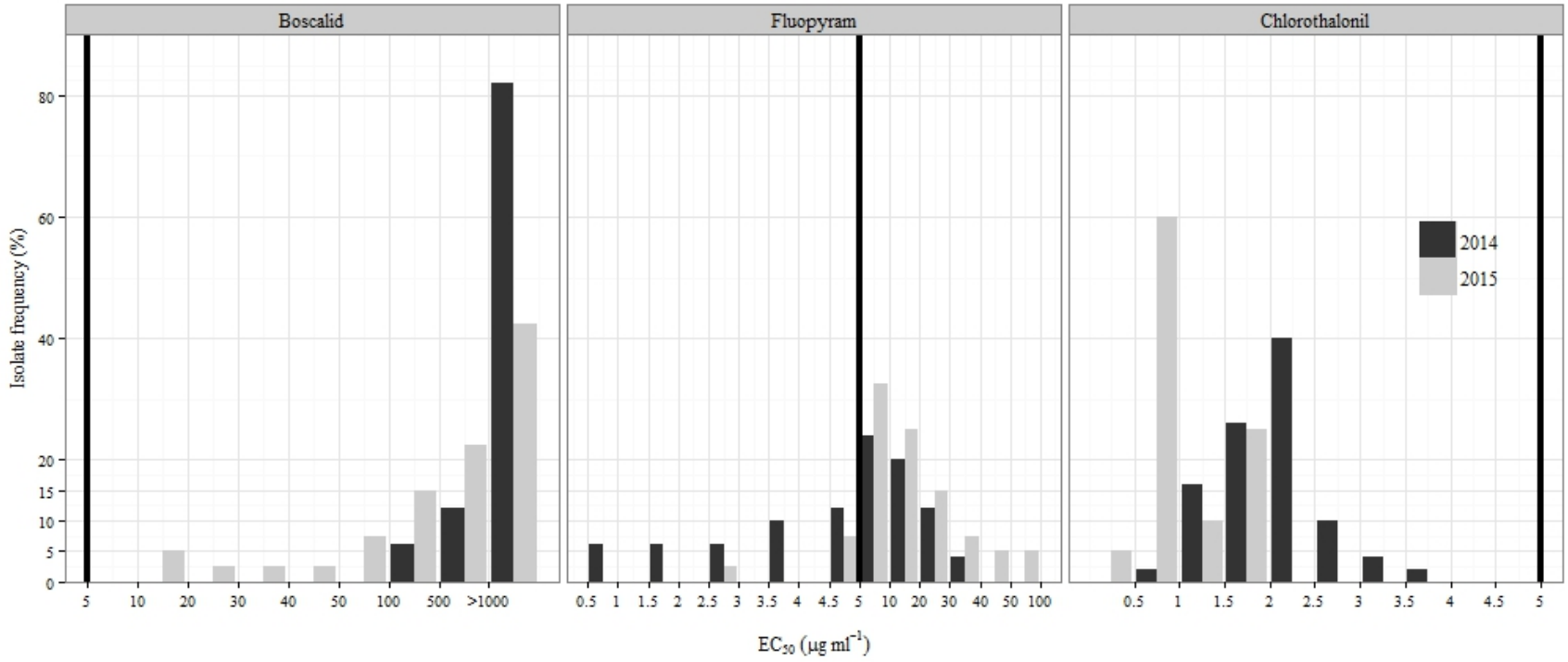

Figure 1. Distribution of 50 A. solani isolates collected in 2015 and 40 isolates in 2014 of sensitivities to boscalid, fluoypram, and chlorothalonil.. Distribution of 40 A. solani isolates in 2014 and 50 isolates collected in 2015 of sensitivities to boscalid, fluoypram, and chlorothalonil. Isolates were collected from research field plots near Miller, Research in Rupert, ID. Effective concentrations which inhibits spore germination by 50% were estimated using in vitro methods compared to non-amended control (EC50 µg ml^{-1}). Line at 5 µg ml^{-1} represents threshold for resistance; bars to the left of line are sensitive and bars to the right of line are resistant

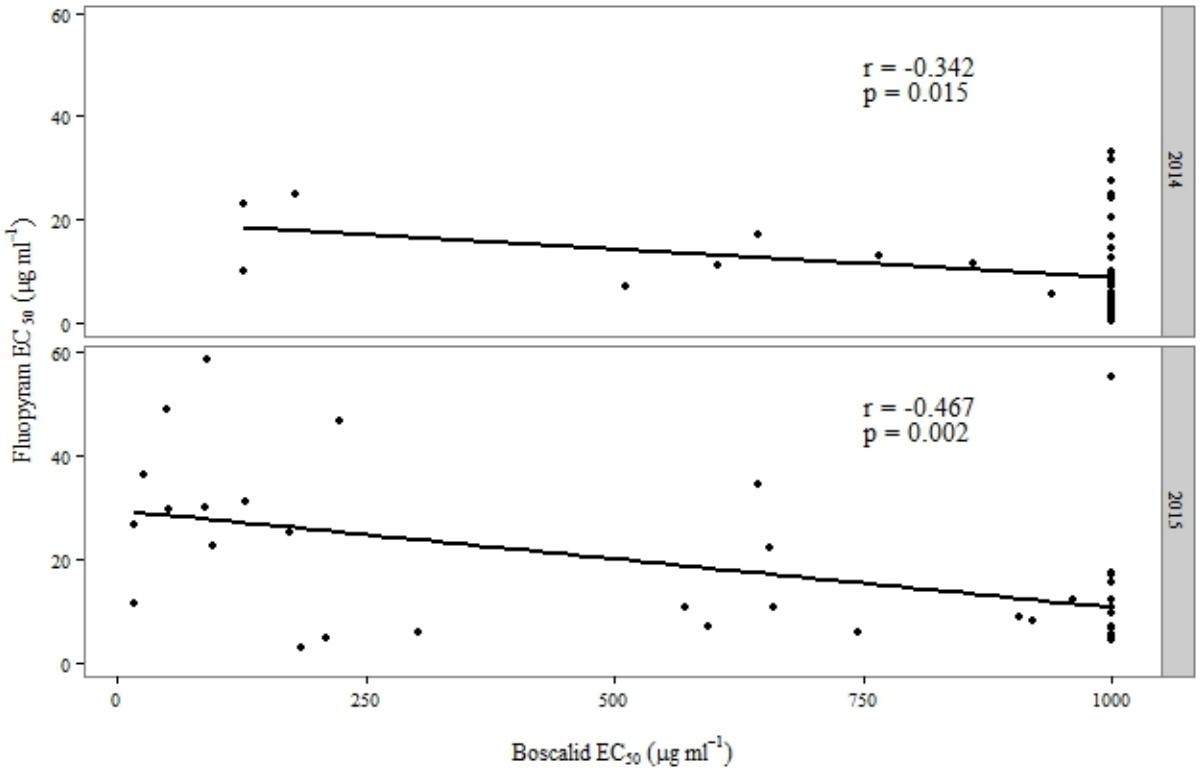

Figure 2. Spearman rank correlation of *in vitro* sensitivities to fluopyram and boscalid to 50 isolates collected in 2014 and 40 isolates collected in 2015.

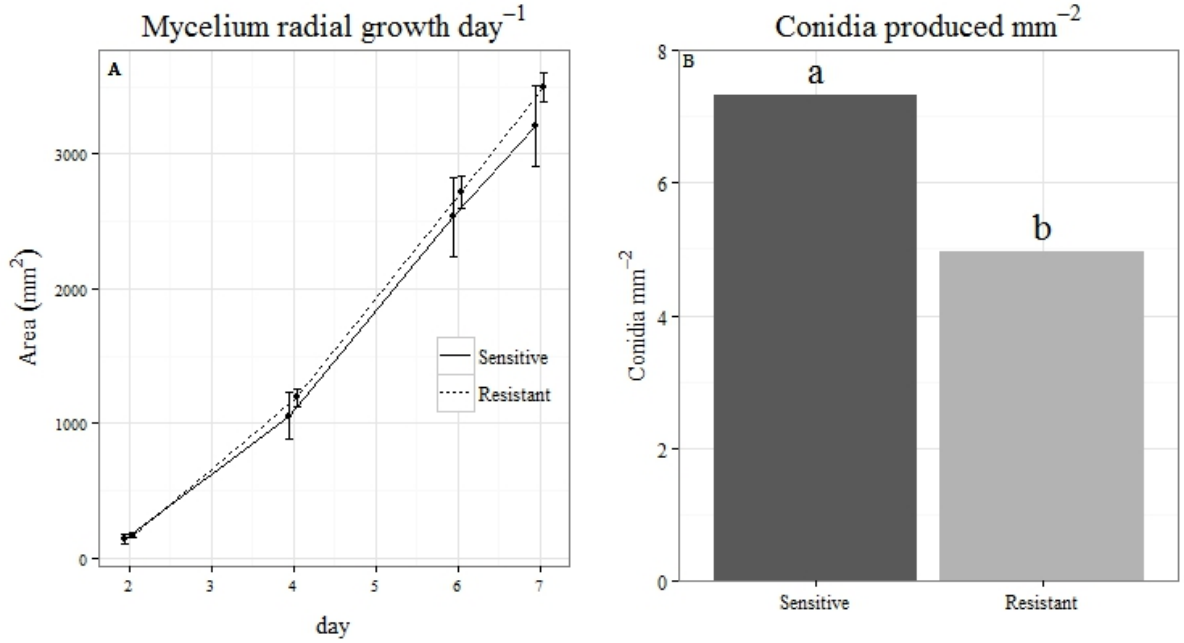

Figure 3. Fitness, determined by conidia production, of 26 sensitive and 64 resistant isolates collected from field plots in 2014 aned 2015 to fluopyram as determined from the *in vitro* effective concentration 50% assays. A - Radial measurements were taken 2, 4, 6, and 7 days after initiation of culture. Error bars represent confidence intervals. Model: Ln Area=day+sensitive+resistant+day2+day*sensitive+day*resistant (P = 0.24). B- Measurement of conidia mm^{-2} (P = 0.052). Letters represent significance.

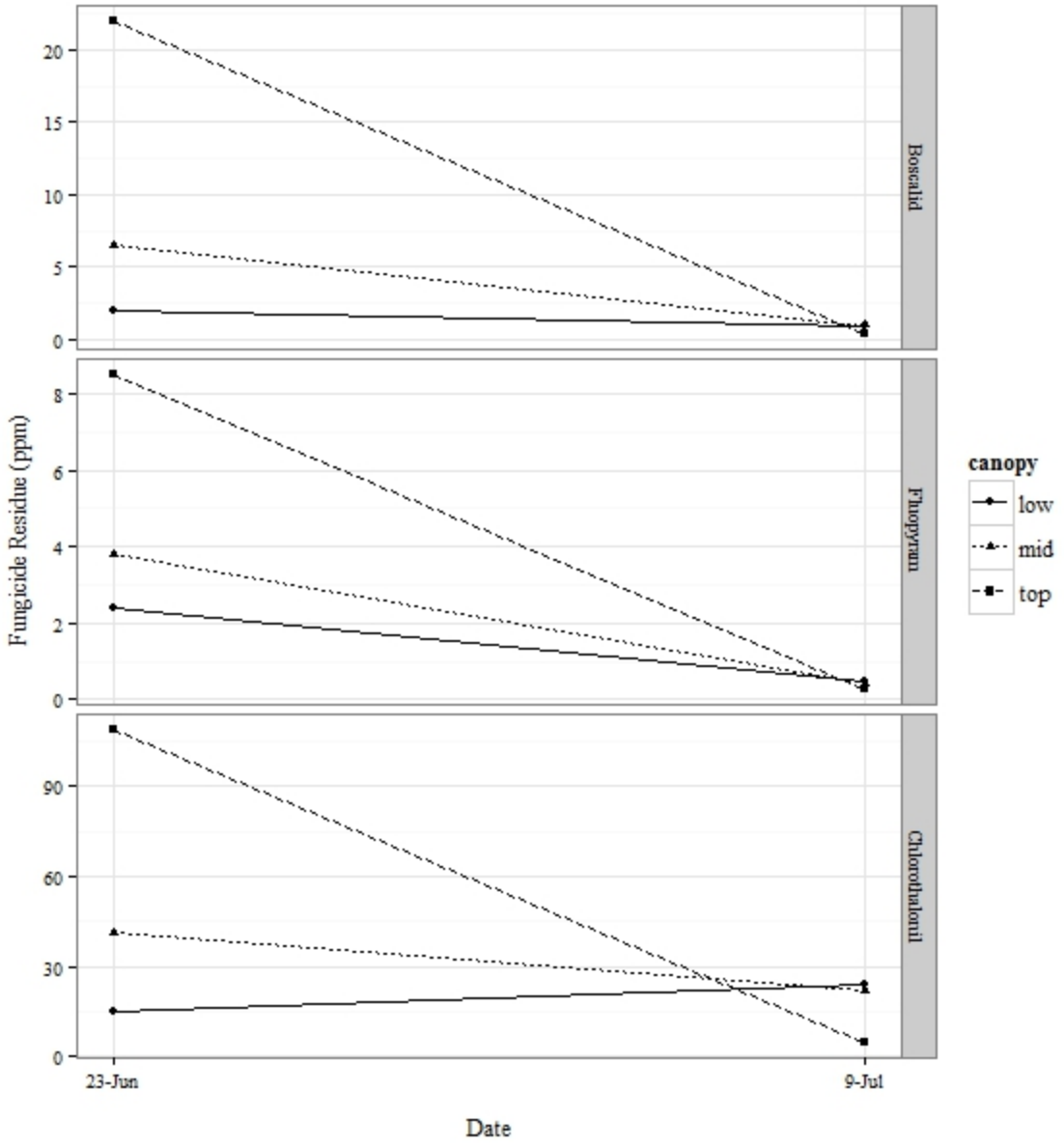

Figure 4. Concentrations of boscalid, fluopyram, and chlorothalonil residues on potatoleaflets at one and 16 sixteen days application. Samples tested for boscalid residue were taken from treatment B-15. Samples tested for fluopyram residue were taken from treatment C-15. Samples tested for chlorothalonil residue were taken from treatment D-15. Fungicides were applied June 23, 2015 and were not applied again for two weeks, July 6, 2015.

CHAPTER 2-Literature Review

INTRODUCTION

Early Blight of potatoes is a fungal pathogen that decreases yields. The causal agent of early blight is *Alternaria solani* (E&M) Sorauer. Different fungicides have been used to control the disease but resistance develops in a matter of years. Boscolid, a succinate dehydrogenase inhibitor (SDHI), showed great efficacy when it was first introduced on the market but 4 years later, evidence of resistance was observed among fields in Idaho (Fairchild et al. 2013; Gudmestad et al. 2013). Fluopyram is another SDHI that was introduced to the market after Boscolid. The two fungicides are in the same group but no cross resistance has been observed to isolates of *Alternaria solani*.

Chlorthalonil is a multi-site inhibitor, also used to control the early blight disease on potatoes. The fungicide is considered a low risk fungicide due to its ability to inhibit multiple enzymes. However, recently a report was made that there was 35%-40% resistance in isolates of *Alternaria solani* in Idaho (Fairchild et al. 2013). The amount of resistance observed from the study is suspect due to the low probability of resistance developing to Chlorothalonil and the methods used to determine resistance.

Therefore the objectives of this research are to (1) determine if populations of *A. solani* collected from areas where Boscolid, Fluopyram, and Chlorothalonil were applied differ in their sensitivity to the respective fungicides; (2) determine if Boscolid confers cross resistance to Fluopyram, and vice versa; (3) determine if there is a fitness penalty for maintaining resistance potential; and (4) identify recommendations of fungicide sprays that minimize the buildup of resistant *A. solani*.

LITERATURE REVIEW

Potatoes

Domesticated plants struggle with the abundance of pests that invade and damage tissue, lowering yields and sometimes causing devastating economic disasters. Since the 1960's pesticides have played a major role in protecting our crops from pests such as insects, fungi, bacteria, and viruses. In 2005, potatoes treated with pesticides had estimated yield loses of 14, 7, 11, and 8% due to pathogens, viruses, insects, and weeds, respectively; without pesticides yield losses were estimated at 75% (Oerke 2006).

Potatoes are an important crop in the United States and the world. It is the number one consumed vegetable in the US, which is the fifth largest producer of potatoes with sales exceeding $3.7 billion (FAO 2014; USDA-NASS 2015). Potatoes are an economically important crop that must be protected from pests because they significantly reduce yields and the US needs to protect the more than 447 thousand metric tons of potatoes distributed throughout the world (FAS, Office of Global Analysis 2013) .

Resistance

Pesticides are an important means to protecting plant products from damage, and in 1960, more than 89 million kilograms of active ingredient were used for pest control. This quantity grew to 287 million kilograms of active ingredient in 1981, but has since decreased to 234 million kilograms in 2008. Farmers in the US spent about $12 billion for pesticides in 2008, of which 80% were applied to corn, soybeans, wheat, and potatoes. Herbicides account for 76% of active ingredient applied to crops in 2008, and insecticides approximated 5.5% of active ingredient. Potatoes received approximately 10% of all active ingredient used in 2008, and almost 10% of that used on potatoes were fungicides (Fernandez-Cornejo et al. 2014).

The use of herbicides has greatly increased due to herbicide-resistant cotton, soybean, and corn, which contains over 90% herbicide resistant varieties. An additional 43 million kilograms of glyphosate were used in 2008 on soybeans, and another 37 million kilograms on corn and cotton as a result of herbicide resistant varieties (Fernandez-Cornejo et al. 2014). With this increased use, resistance has been found among crops that have been treated exclusively with glyphosate (Culpepper et al. 2006; Heap 2014; VanGessel 2001). These new biotypes have needed up to 7 times the listed rate to decrease the biomass by 50%. The use of Bt corn and cotton has decreased the use of insecticides; however, aphids and the Colorado potato beetle have shown resistance to imidicloprid. Other studies have found insects have gained resistance to different insecticides despite the reduction in the amount of insecticides applied (Mota-Sanchez et al. 2006; Su et al. 2014; Weichel and Nauen 2003).

Fungi, like weeds and insects, also develop resistance to fungicides, particularly those that are used extensively, and they do it at a faster rate weeds. Fungicides act as protectants to the host from the pest and/or can be curative once the fungi has colonized the host. Fungicides can translocate within the plant (systemic) or can be stagnate on the leaf surface (contact). There are many different fungicidal classes that are determined by their mode of action; the Fungicide Resistance Action Committee put together a code, based on the mode of action and targeted site of the fungicide (FRAC 2015). Each mode of action has different target sites; there are also different chemical groups for each target site. The Fungicide Resistence Action Committee also helps to educate growers about the risks of fungicide resistance developing and how to protect against that development.

Fungicide resistance levels defined in a lab do not always correlate to the concentrations of fungicides found in the field. Resistance in this study, and other previous studies, identified

resistant isolates when germination occurred after exposure to 5 or more μg ml^{-1} of fungicide (Gudmestad et al. 2013; Kim and Xiao 2010; Yin et al. 2011). Field residues are subjected to a number of variables such as irrigation, photo degradation, and new plant growth that diminish fungicide concentrations within the upper and middle canopies. The observed trend in residues of boscalid, fluopyram, and chlorothalonil followed similar trends observed by Geary et al. (1999) in that fungicide concentrations were higher in all canopy levels immediately after application but decreased in the upper to mid-levels of the canopy prior to another application. Geary et al. (1999) also observed that as residues diminished, percent disease severity also increased. The loss of effective fungicide concentrations between two applications may be a factor in the development of resistance as not all isolates are exposed to lethal rates of fungicide. If resistance is to be managed, then an understanding of fungicide persistence within a canopy is necessary to maintain lethal concentrations.

Fungicides in the 1960's were predominantly copper based or multi-site inhibitors (MSI), meaning they disrupted several different pathways of the pathogen by inhibiting general enzymes. The MSI fungicides from the 1960's had limited efficacy and were considered harmful to humans and to the environment, resulting in the search for single-site mode of action and more efficacious fungicides. Single mode of action fungicides were developed to target a specific metabolic site and physiological pathways of the pathogen. Now there are many fungicides each with a unique mode of action and their chemical classes along with information detailing the risk of resistance (FRAC 2015). Fungicides are grouped according to their mode of action, five main groups that have been used over the past 30 years are 1- benzimidazoles, 2- demethylation inhibitors (DMI), 3- Quinone outside inhibitors (QoI), 4- succinate dehydrogenase inhibitors (SDHI), and 5- multi-site inhibitors (MSI).

Benzimidazoles helped the transition from these multi-site and copper based fungicides to single-site inhibitors during the late 1960s. This group targets microtubule formation and inhibits cell division (Clemons and Sisler 1971; Davidse 1986; Davidse and Flach 1977; Quinlan et al. 1980). Unfortunately, resistance to the benzimidazoles was detected within a few years after introduction. Powdery mildew of cucurbits had been controlled by benzimidazoles for many years; however in 1969, there were reports of resistance developing among some isolates (Schroeder and Provvidenti 1969). Benzimidazoles have also been used on potato tubers to control *Helminthosporium solani* but resistance was found soon after they were introduced (Hide et al. 1988; Jellis and Taylor 1977). *Monilina fruticola,* the causal agent of brown rot in apricot and prune trees, has also developed resistance to benzimidazole fungicides. Brown rot accounted for $2.82 million in annual loss and expenses in 1963 (Michailides et al. 1987). Fungicides of this group have diminished in use since resistance was identified and the control of pathogens is expensive but becomes more expensive when resistance develops and higher rates or more frequent applications are needed for control. Observing the cost of control and annual losses from *M. fruticola* demonstrates how resistance can be a serious problem. Interestingly, as applications decreased studies have shown that frequency of resistant isolates decreased while non-resistant isolate frequency increased (McGrath et al. 1996). Demonstrating there is a fitness expense to the pathogens for possessing resistance capabilities.

Demethylation inhibitors were also developed in the 1960's and provided a broad spectrum of control against many different pathogens in a variety of crops (Scheinpflug 1988). The DMI's mode of action creates leaks in the fungal membranes; the fungicide binds to an enzyme, keeping 24-methylenedihyrolanosterol from progressing on to ergosterol (Vanden Bossche et al. 1987). Resistance to the DMI mode of action was first observed on cucurbits

infected with powdery mildew (Huggenberger et al. 1984). Continued use of these fungicides resulted in more fungi becoming resistant: *Botryosphaeria dothidea,* the causal agent of panicle and shoot blight, and *Blumeriella jaapii*, the causal agent of cherry leaf spot (Proffer et al. 2006). According to the Fungicide Resistance Action Committee, DMI fungicides are classified as medium risk for resistance development.

Quinone outside inhibitors (QoI) have good efficacy on a number of different pathogens and were developed from two fungi: *Strobilurus teacellus* and *Oudemansiella mucida*. The common name for QoIs has become strobilurins (Knight et al. 1997). The target site for strobilurins is cytochrome b protein in Complex III of the mitochondria; the fungicide inhibits electron flow between cytochrome C_1 and b, effectively inhibiting NADH oxidation (Becker et al. 1981; Von Jagow et al. 1986). In the United States, the fungicide azoxystrobin, a strobiluron fungicide, was registered in 1999 for potato use and within a few years resistance had developed in *Alternari solani*, the causal agent of early blight. Azoxystrobin was used in potato fields to control early blight but reports of reduced efficacy were reported as early as 2000. Isolates of *A. solani* were collected across several states from 1998-2001 and evaluated for resistance. A shift from sensitive to resistant *A. solani* was observed across all years but was more pronounced in 2001 (Pasche et al. 2004). Other *Alternaria* species have also developed resistance to QoIs such as *A. alternata, A. tenuissima,* and *A. arborescens* (Ma et al. 2003). Researchers in Japan identified resistance to QoIs in powdery mildew (*Podosphaera fusca* (Fr.)) and downy mildew (*Pseudoperonospora cubensis* (Berk. & M. A. Curtis) Rostovzev) on cucumber (Ishii et al. 2001). Due to resistance issues, the QoIs are not used as frequently on the many crops that initially used them.

Succinate dehydrogenase inhibitors were also developed in the 1960's and had good control of many plant pathogens. The mode of action of SDHI fungicides disrupts cellular respiration in the cell by targeting the succinate dehydrogenase complex (Avenot and Michailides 2010b; White and Thorn 1975). Laboratory mutation experiments trying to understand the mode of action reported resistance development to SDHIs soon after the fungicide was developed (Ben-Yephet et al. 1974; Georgopoulos and Sisler 1970). Field studies of *Ustilago maydis* later confirmed the laboratory resistance development (Leroux and Berthier 1988). Field resistance has also been reported in *Monilini fructicola, Botrytis cinerea, Alternaria solani,* and other fungi (Amiri et al. 2010; Avenot and Michailides 2007; Leroux et al. 2010).

The fifth group of fungicides MSIs, have unique modes of action for each of the different chemical classes but because they target multiple cites so they are grouped as MSIs. Since MSIs target fungi at multiple sites of inhibition, resistance development is considered low. Research has shown some evidence of resistance, but only in small percentages of pathogen populations. A study conducted at Cornell University with isolates of *Phytophthora infestans* collected in Mexico, showed variable resistance levels in a couple of isolates (Sujkowski et al. 1995). A small amount of resistance was also found in *Botrytis cinerea* (Zhang et al. 2009). The low amounts of resistant isolates are not enough for concern but monitoring of resistance should continue.

Site-specific fungicide groups, such as the four mentioned above, have improved pathogen control compared to MSIs, but resistance is becoming more prevalent and reducing site-specific efficacy. Fungicide resistance in potatoes is a problem for potato producers and if site-specific fungicides are ineffective then potato yields will go down. Potatoes are susceptible to many fungal pathogens and require multiple fungicide applications for a healthy crop. The

frequency of fungicides applied to potatoes has increased from 24% in the late 1960's to 85-95% in 2008 (Fernandez-Cornejo et al. 2014). One of the many fungal pests of potatoes is *Alternaria solani* (E&M) Sorauer, the causal agent of early blight. *Alternaria solani* can reportedly form resistance rather quickly (Pasche et al. 2004), which is problematic because the pathogen can reduce yields by 50% (Harrison and Venette 1970; Neergaard 1945). An understanding of resistance development is necessary to keep labeled fungicides effective against *Alternaria solani* and stop the loss of these important production tools.

Early Blight Disease

Alternaria solani was first identified in 1882 from dying potato leaves by Ellis and Martin and was called *Macrosporium solani;* it was later changed to the genus *Alternaria* by Jones (Ellis and Martin 1882; Vermont Agricultural Experiment Station. 1888). *Alternaria solani* is identified by long multi-celled conidia with tails that are just as long as or longer than the conidia. Potato plants develop lesions where infected, which appear brown and circular, about 1-2 mm in diameter. As the lesions grow on potato leaves concentric rings emerge giving the lesion a "bull's eye" look. The edges of the lesion may be surrounded by a yellow halo. The pathogen can overwinter on infected host debris or on volunteer hosts and weeds of the Solanacea family (Rands 1917). *Alternaria solani* is a hardy fungus and can survive through hot dry summers and cold, wet winters. The conidia germinate and infect plants when the weather is warm and humid (24-29°C) (Bashi and Rotem 1974). The stomata are the main entrance of the germ tubes into the epidermis. Symptoms usually occur first in older leaves under warm, moist climates. Wind is the principle mode of dissemination but rain can also spread conidia. *Alternaria solani* is a polycyclic pathogen meaning it will go through multiple cycles throughout the growing season where conidia are produced repeatedly (Kemmitt 2013). *Alternaria solani*

belongs to the phylum Ascomycota, order Pleosporales, family Pleosporacea (Ainsworth 2001). The genus *Alternaria* was first established in 1817 by Nees (Rotem 1994b).

Alternaria fungi have reportedly gained resistance to a number of fungicides (Avenot and Michailides 2007; Dry et al. 2004; Fairchild et al. 2013; Gudmestad et al. 2013; Iacomi-Vasilescu et al. 2004; Ma et al. 2003; Rosenzweig et al. 2008). Resistance development in *A. solani* to fungicides from the modes of action groups QoI, SDHI, and MSI have become a problem to many crops but are posing a serious concern to potato growers trying to manage early blight. It is no longer viable to apply a fungicide with the same mode of action over and over again, instead growers must alternate modes of action and monitor for cross-resistance development. Cross-resistance is when a pathogen develops resistance to the effects of a fungicide because of exposure to a related class of fungicides. Cross-resistance does not occur between modes of action, thus the need to alternate, but it can develop between or among classes of fungicides within a mode of action. *Alternaria* spp. have been observed to develop cross-resistance among classes of fungicides within fungicide mode of action but not between the SDHI or MSI groups (Amiri et al. 2014; Fairchild et al. 2013; Gudmestad et al. 2013; Hildebrand et al. 1988; Ishii et al. 2001).

Alternaria solani has been controlled in potato production in recent years with Boscolid (Endura; BASF Corporation, Agricultural Products, Research Triangle Park, NC), Fluopyram (Luna Privilege, Bayer Crop Science, Durham, NC), and Chlorothalonil (Bravo, Syngenta Crop Protection, LLC, Greensboro, NC). Resistance has been observed to these three fungicides (Fairchild et al. 2013; Gudmestad et al. 2013; Pasche et al. 2004). Boscolid and Fluopyram are SDHI fungicides but belong to different fungicide classes even though they have the same mode

of action. Chlorothalonil is a MSI fungicide, one report of resistance has been documented but it is unclear if resistance is growing (Fairchild et al. 2013).

The SDHI fungicide, Boscolid, was registered in 2005 for control of early blight on potatoes. Boscolid proved to have good efficacy controlling early blight and became a popular replacement when resistance was observed to other groups of fungicides (Pasche et al. 2005). Soon after Boscolid was introduced resistance was detected and became prevalent in several states across the US. Of Isolates collected in 2010-2011, 75% demonstrated EC_{50} levels of 5 μg/ml and being labeled resistance to Boscolid (Gudmestad et al. 2013). Boscolid is in the chemical class pyridine-carboximides and binds, or docks to the succinate dehygrogenase (SDH) subunits of the mitochondrial respiratory chain (complex II). Different mutations of the SDH genes cause different levels of resistance to the fungicide by changing the shape of the SDH enzyme (Fraaije et al. 2012; Scalliet et al. 2012).

Fluopyram was registered for potato use in 2012 to control early blight. This SDHI fungicide belongs to the chemical class pyridinyl-ethyl-benzamides, which is different structurally to Boscolid (table 1) (Fig. 1). Studies have demonstrated that low levels of resistance has formed to Fluopyram while some of the same isolates are resistant to Boscolid (Amiri et al. 2014). The mutations of the SDH enzyme that cause resistance to Boscolid do not necessarily render resistance to Fluopyram, so cross-resistance development in Fluopyram should not be due to *Alternaria solani* exposure to Boscolid. The docking of fungicide is a key factor in the toxicity of a fungicide to a pathogen and Fluopyram docks to the SDH protein in a different manner than Boscolid (Fraaije et al. 2012). After three years of application to commercial potato fields, resistance to Fluopyram has not been observed in high frequencies but resistance suggest a cross-resistance effect due to Boscolid, or resistance is building due to mutation effects in the SDH

genes. Currently, the low levels of resistance in Fluopyram has only been explained by changes in the docking site of the molecule to the SDH enzyme and there are still some questions regarding its true effects (Fraaije et al. 2012; Scalliet et al. 2012).

Chlorothalonil is a MSI that effectively protects potatoes from the early blight disease (Holm et al. 2003). The plant is protected as UV light breaks down Chlorothalonil, producing derivatives that inhibit thiol-dependent enzymes in the fungus (Khan and Akhtar 1983; Tillman et al. 1973). The derivatives cause multiple reaction sites that make resistance development more difficult, thus Chlorothalonil is considered to be a low risk fungicide for resistance development by FRAC. However, studies with *Phytophthora infestans* and *Botrytis cinerea* have reported limited resistance to Chlorothalonil in a few isolates (Sujkowski et al. 1995). A report of *Alternaria solani* resistance to Chlorothalonil has caused concern with potato growers because Chlorothalonil has had excellent control of early blight and when tank mixed with other fungicides it helps to reduce resistance build-up to fungicides such as Boscolid and Fluopyram (Fairchild et al. 2013).

Boscolid and Fluopyram are great controllers of early blight on their own but FRAC maintains that all single-site fungicides should be tank mixed with other fungicide groups. In potatoes these fungicides are often mixed with Chlorothalonil. Field trials have shown that efficacy of Boscolid and Fluopyram go up when mixed with Chlorothalonil. However, a mix of Fluopyram and Chlorothalonil demonstrated better results than Boscolid and Chlorothalonil (Horsfield et al. 2010; Miller 2012).

Monitoring for resistance to Boscolid, Fluopyram, and Chlorothalonil are necessary because of the importance to minimize early blight in potatoes. However, the application of these three fungicides selects *A. solani* populations resistant to the fungicides. The resistant trait within

the fungus gives it a fitness—ability to survive different environments—advantage that is passed on to progeny, and eventually the fungal population is resistant. If the build-up of resistance to Boscolid, Fluopyram, and Cholorothalonil could be better understood by knowing the importance of cross-resistance and the time frame in which resistant populations predominate, then we could increase the longevity of these fungicides. Some research has suggested that there is not a fitness cost as a result of resistance (Chapara et al. 2011; Karaoglanidis et al. 2011). However, in other studies fitness costs have been observed in resistant pathogens (Billard et al. 2012; Iacomi-Vasilescu et al. 2008). A reversion back from resistant to susceptible isolates is ideal as was seen among isolates of *Sphaerotheca fuliginea* of cucurbits. After 20 years *S. fuliginea* was more sensitive to benomyl after not being used (McGrath et al. 1996). Fitness and cross-resistance both have to do with the mutations causing resistance in pathogens (Mallik et al. 2014; Miles et al. 2014). Research done by Miles et al. (2014) observed how a mutation in the H133R gene of succinate dehydrogenase subunit D can result in resistance to boscalid and fluoypram. Mutations my not affect one single gene but multiple genes making it difficult to find fitness costs. Factors of mutations also need to be looked into for better understanding of resistance and fitness. If the timing and alteration, or tank mixing, of fungicides were developed so that non-resistant isolates predominate the population then the disease could be better controlled and our current fungicides could be used effectively for many years without the development of new fungicides.

Therefore the objectives of this research are to (i) determine if populations of *A. solani* collected from areas where Boscolid, Fluopyram, and Chlorothalonil were applied differ in their sensitivity to the respective fungicides; (ii) determine if Boscolid confers cross resistance to Fluopyram, and vice versa; (iii) determine if there is a fitness penalty for maintaining resistance

potential; and (iv) identify recommendations of fungicide sprays that minimize the buildup of resistant *A. solani*.

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