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Assessing fungicide resistance in populations of *Alternaria* in Idaho potato fields

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ABSTRACT

Early blight, caused by the fungus *Alternaria solani* and brown leaf spot, caused by *Alternaria alternata*, are important diseases of potato crops in Idaho. In recent years growers have reported a reduction in efficacy of fungicides traditionally used in the past decade to control early blight. In 2009, a collection of *A. solani* 39 isolates were screened for resistance to azoxystrobin, pyraclostrobin, boscalid and famoxadone. Fungicide sensitivity testing was done using spiral plate dilution gradients. Results showed that of 39 isolates screened, all were resistant to azoxystrobin and three were resistant to boscalid. None were resistant to pyraclostrobin or famoxadone. In summer 2010, more isolates were collected (9 *A. alternata* and 26 *A. solani*) and the survey was expanded to include more fungicides with four different modes of action that targeted succinate dehydrogenase (SDH), methionine biosynthesis, mitochondrial respiration and multi-site contact activity. New isolates of *A. solani* and *A. alternata* were also collected from two additional sites. The results showed that 57% of the isolates were resistant to boscalid as well as an average of 63% of the isolates being resistant to the strobilurin fungicides. Seven and 15% of isolates were resistant to penthiopyrad (an SDH inhibitor), and pyrimethanil (a methionine biosynthesis inhibitor), respectively. However, none of the isolates were resistant to fluopyram (an SDH inhibitor) or a mixture of fluopyram and pyrimethanil. Although there appears to be cross resistance developing in *Alternaria* spp. to some of the new SDH inhibitors like penthiopyrad, others such as fluopyram are still showing limited to no resistance development in *Alternaria* spp. in Idaho.

1. Introduction

Early blight caused by the pathogen *Alternaria solani* Sorauer and brown leaf spot caused by *Alternaria alternata* (Fries.) Keissl are economically important to potato crops in Idaho, and around the world, as they cause defoliation later in the growing season and reduce potato yield (Franc and Christ, 1981; Rotem, 1994). Yield losses can be reduced with protective, timely fungicide applications (Douglas and Groskopp, 1974; Harrison and Venette, 1970; Weingartener, 1974). Currently, there are a wide variety of fungicides to choose from for the control of early blight. However, the strobilurin or quinone outside inhibitor (QoI) fungicides are often favored because they offer broad spectrum protection against a wide range of fungal and oomycete diseases, have reduced environmental impact, and reduced toxicity to mammals and bees compared with conventional protectant fungicides (e.g. chlorothalonil, mancozeb, and mefenoxam) used to control early blight (Rosenzweig et al., 2008a).

In recent years there have been numerous reports of a reduction in efficacy of QoI fungicides traditionally used to control early blight (Pasche and Gudmestad, 2008; Rosenzweig et al., 2008a, 2008b). Resistance to the QoIs has been characterized in many plant pathosystems including *A. alternata* on apples (Ishii, 2008), *Botrytis cinerea* on strawberries (Markoglou et al., 2006), and *Erysiphe necator* on grapes (Wong and Wilcox, 2002). In many fungi, resistance has been attributed to the presence of the G143A mutation, which involves the substitution of glycine by alanine at the amino acid position 143 (Sierotzki et al., 2000; Ishii et al., 2001; Kim et al., 2003). However, in *A. solani* a reduction in fungicide sensitivity has been attributed to the F129L mutation, which is the substitution of phenylalanine with leucine at position 129 (Pasche et al., 2004, 2005).

Little is known about the prevalence of *Alternaria* isolates with reduced sensitivity to the QoIs in Idaho as there has only been one report of it previously and that was on a very limited number of isolates collected in 2005 (Pasche and Gudmestad, 2008). Thus, in

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2009, a survey was carried out to screen *A. solani* isolates for resistance to the QoI fungicides. Isolates were also screened for resistance to the carboxamide fungicide boscalid (Fig. 1). Unexpectedly, it was discovered that 15% of *A. solani* isolates were resistant to boscalid (Wharton et al., 2012). Thus in 2010, the fungicide resistance survey was expanded to cover the four fungicide resistance action committee (FRAC) groups of fungicides commonly used in Idaho for the control of *Alternaria* diseases on potato (FRAC Code list, 2011). The FRAC groups tested included the fungicides fluopyram, penthiopyrad and boscalid (group 7), pyrimethanil (group 9), trifloxystrobin, pyraclostrobin, fenamidone, famoxadone, azoxystrobin and picoxystrobin (group 11), and chlorothalonil (group M5).

Pyrimethanil and other anilino-pyrimidine fungicides (FRAC group 9) affect methionine biosynthesis and resistance has been reported in *B. cinerea* on grapes (Sergeva et al., 2002) and *Penicillium expansum* on apples (Li and Xiao, 2008) but site specific mutations have not been identified. Boscalid and other succinate dehydrogenase inhibitor (SDH inhibitor) fungicides (FRAC group 7) also inhibit fungal respiration. Resistance in *A. alternata* on pistachio has been associated with mutations in three subunits of succinate dehydrogenase (i.e. *AaSDH-B*, *AaSDH-C*, and *AaSDH-D*). Two single nucleotide mutations in SDH-B (H277Y and H277R) have been found in resistant isolates (Avenot et al., 2008). Mutations in the membrane anchoring subunits SDH-C (H134R) and SDH-D (H133R and D123E) have also been identified (Avenot et al., 2009).

The goals of this study were to determine the prevalence of *Alternaria* isolates with reduced sensitivity to the QoI fungicides and to determine the frequency of resistance in populations of *A. solani* and *A. alternata* to the other FRAC groups of fungicides currently used in Idaho to control early blight and brown leaf spot.

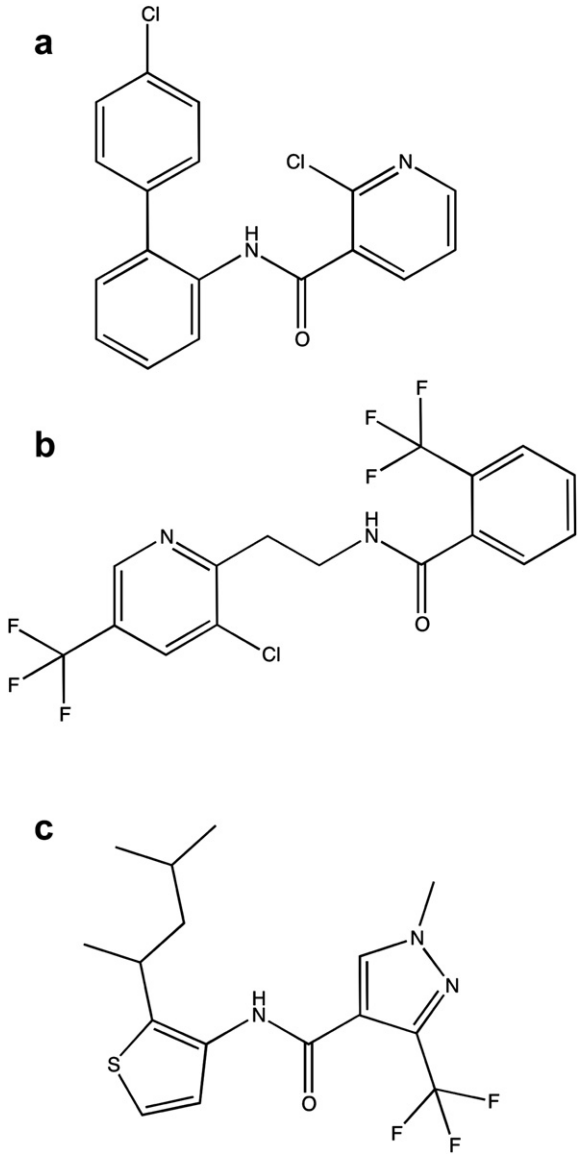

Fig. 1. Chemical structures of succinate dehydrogenase inhibitor fungicides (FRAC group 7) including: a) boscalid (2-chloro-*N*-(4′-chlorobiphenyl-2-yl)nicotinamide) b) fluopyram (*N*-{2-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethyl}-α,α,α-trifluoro-*o*-toluamide) and c) penthiopyrad ((*RS*)-*N*-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide).

2. Materials and methods

2.1. Sampling

In 2009 and 2010, early blight symptomatic leaf samples were collected from fields in the main potato growing regions in southern Idaho, including areas in and around Aberdeen, Parma, Rupert, and in northern Idaho from Bonners Ferry. From these a total of 74 isolates were obtained, 39 in 2009 and 35 in 2010. Of these 74 isolates, 41 were obtained from Aberdeen, 15 from Bonners Ferry, 6 from Parma and 12 from Rupert.

2.2. Fungal isolation and morphological and molecular identification

To obtain *Alternaria* isolates from leaves, small pieces of leaf tissue (5 × 5 mm) were taken from the center of the early blight lesions using a sterile scalpel and streaked across the surface of a thin layer (3 mm) of tap water agar (TWA) using sterile tweezers. Plates were incubated at 25 °C to allow conidia to germinate. Single germinated *A. solani* or *A. alternata* conidia were transferred, with the aid of a dissecting microscope, to acidified potato dextrose agar (PDA; Difco, Detroit, MI) containing 500 μL per liter glacial acetic acid, and incubated in the dark at 25 °C. Germinated conidia were identified based on conidial morphology. Conidia of *A. solani* can be distinguished from *A. alternata* as they are ellipsoid to oblong and taper to a long beak, which is usually as long as the conidial body. The identity of cultures grown from single conidia was determined by colony and conidial morphology. These were confirmed using molecular taxonomy techniques which involved sequencing the internal transcribed spacer regions (ITS1 and ITS2) using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′) as described in Sambrook et al. (1989). Single spore cultures were allowed to grow an additional week, after which time two of the cultures from each original leaf were selected and categorized for sporulation and testing.

2.3. Sporulation techniques

After the test cultures were selected, two subcultures of each were made on PDA, and incubated in complete darkness for at least three weeks at 21 °C. To obtain optimal sporulation, these cultures were subjected to five minutes of daylight once a week (Rotem, 1994). After the three week period cultures were taken out of the dark for use in fungicide resistance experiments. In the fungicide resistance experiments, conidial suspensions were made as follows. Fungal cultures were flooded with 5 mL of sterilized deionized water (SDW) and conidia were then dislodged from the media using a bent glass rod which was gently scraped across the surface of the media. The conidial suspension was then

filtered through two layers of sterile cheesecloth and placed in a 50 mL sterilized beaker. Each conidial suspension was then checked using a hemocytometer and adjusted to 1.0–2.0 $\times$ 10^4 spores/ml.

2.4. Screening fungicide sensitivity

All fungicides tested were commercial formulations, with some having more than one active ingredient (Table 1). In each experiment three fungicides were tested against five *Alternaria* isolates. Fungicide stock solutions were prepared at a concentration of 10,000 mg/L in distilled water. In a comparison study, isolates that were grown on non-antibiotic amended PDA plates showed no significant difference in colony growth compared to those grown on antibiotic amended plates. Thus, in order to reduce bacterial contamination, 51 PDA plates amended with rifamycin and ampicillin (15 cm in diameter) were used per experiment. Initially, in the fungicide screening the spiral gradient dilution method (Forster et al., 2004) was compared to the classical poisoned agar plating techniques on a subset of *A. solani* isolates and similar results were observed for both assays. In 2009, in the tests for QoI resistance no differences were found between isolates grown on media with or without the addition of salicylhydroxamic acid (SHAM) so in the 2010 QoI screening assays SHAM was not used. Similar percentages of resistant isolates were identified in all years (data not shown). Each test fungicide was spread across the media (15 cm plates) using an Eddy Jet 1700 spiral plater (IUL Instruments, Barcelona, Spain). The highest concentration (10,000 mg/L) was laid down in the center of the media and a fungicide gradient which decreased exponentially from the center to the edge of the plate was created (Supplementary Fig. 1). To calculate 10,000 mg/L for mixed fungicides (more than one active ingredient) the most concentrated active ingredient was used. Control plates were amended with SDW only. The spiral plating methods were adapted from Forster et al. (2004).

Conidial suspensions (10 μL) were applied to each fungicide amended plate using a micropipette in 3 lines creating a T shape. A colony counter grid (IUL Instruments) printed on a transparent sheet was placed underneath each 15 cm plate and used as a guide to lay down the conidial suspensions on the media. Using a sterile disposable plastic pestle, the conidial solution was spread along the plate position lines several times. Plates were then incubated for four days in the dark at 18°C. All fungicide by isolate experiments were carried out three times.

2.5. Assessing fungal growth

Fungal growth on fungicide amended plates was assessed after 4 days by image analysis. Plates to be assessed were placed face down on a flat bed scanner (Epson Perfection V500 PHOTO; Epson America Inc., CA, USA) and an image was captured, imported into Photoshop CS5 (Adobe Inc., CA, USA), and saved in the TIFF file format with LZW file compression. All images were captured at 300 pixels per inch, which gave a final image that was 2550 pixels wide by 3509 pixels high. Two images per treatment were captured, one of the bottom of the plate and one of the top of the plate. The plates were scanned against an ambient black background so that images were formed from light reflected off the surface of the media. To ensure consistency between the images, 50 ml of PDA media was used in each Petri dish so that the distance between the scanner surface and media in the plate was the same throughout all experiments. This was important to ensure that the brightness of the images were consistent throughout the experiment.

Images were analyzed in Photoshop CS5 using the ruler tool. The analysis scale was set to 119 pixels which equaled one centimeter. This was determined by a ruler that was scanned alongside each test plate. Half maximal effective concentrations (EC_{50}) were calculated by measuring the amount of growth at the point at which 50% growth occurred compared to the control plates. The point on the treatment plate at which this same amount of growth occurred was recorded and the fungicide concentration at this point was determined. This concentration was the EC_{50} concentration for that isolate. If an EC_{50} value for an isolate could not be determined, because it was above the threshold compared to the control plate, the isolate was considered to be resistant to the test fungicide.

Table 1
Chemical and trade names, manufacturer, FRAC group, chemical class, mode of action, target site and FRAC code of fungicides used in the *Alternaria* trials. Based on individual fungicide labels and fungicide resistance action committee (FRAC) website (http://www.frac.info).

FRAC group	Chemical name	Fungicide name	Manufacturer	Maximum rate[a]	Chemical group	Mode of action	Target site and code
7	Boscalid	Endura	BASF, Research Triangle Park, NC	0.73 L/ha	Pyridine-carboxamides	Respiration	C2: complex II: succinate-dehydrogenase
	Fluopyram	Luna	Bayer CropScience, Research Triangle Park, NC	N/A[b]	Pyridinyl-ethyl-benzamides		
	Penthiopyrad	Vertisan	DuPont Crop Protection, Wilmington, DE	1.75 L/ha	Pyrazole-carboxamides		
7 & 9	Fluopyram + pyrimethanil	Luna Tranquility	Bayer CropScience	0.82 L/ha	Pyridinyl-ethyl-benzamides & anilino-pyrimidines	Respiration & amino acids and protein synthesis	C2: complex II: succinate-dehydrogenase & D1: methionine biosynthesis
11	Trifloxystrobin	Gem		0.28 L/ha	Oximino acetates	Respiration	C3: complex III: cytochrome *bc*1
	Fenamidone	Reason 500 SC		0.60 L/ha	Imidazolines		
	Pyraclostrobin	Headline	BASF	0.88 L/ha	Methoxy-carbamates		QoI-fungicides
	Picoxystrobin	Aproach	DuPont Crop Protection		Methoxy-acrylates		
	Famoxadone + cymoxanil	Tanos		0.44 L/ha	Oxazolidine-diones		
	Azoxystrobin	Quadris	Syngenta Crop Protection, Greensboro, NC	1.13 L/ha	Methoxy-acrylates		
9	Pyrimethanil	Scala SC	Bayer CropScience	0.51 L/ha	Anilino-pyrimidines	Amino acids and protein synthesis	D1: methionine biosynthesis
M5	Chlorothalonil	Echo		1.75 L/ha	Chloronitriles (phthalonitriles)	Multi-site contact activity	Multi-site contact activity

[a] Maximum rate per application.
[b] Luna is not registered for use on potatoes. Only Luna Tranquility (fluopyram + pyrimethanil) is registered for use on potato at this time.

2.6. Statistical analysis

All statistical analyses were performed with the JMP statistical computer program (SAS Institute Inc., Cary, NC, USA) and SIGMAPLOT version 10 (SYSTAT Software Inc., Chicago, IL, USA). A one way analysis of variance was conducted to analyze the effect of the isolates locations for each individual fungicide. Additionally, a subset of isolates (collected from Aberdeen, Rupert and Parma) that were tested for all 12 fungicides (Table 1) were analyzed using Tukey's HSD after checking for normality and equality of variance. Statistical differences were determined by a two factor ANOVA ('Isolate', 'Fungicide' and 'Isolate × Fungicide'). Multiple experiments were considered replicates as no statistically significant effect of fungicide sensitivity was observed between experiments ($P < 0.05$). Experimental values were pooled to calculate means and standard error.

3. Results

3.1. Resistant isolates of Alternaria to various fungicides were identified in all sampling locations in both years

In order to assess fungicide resistance in *Alternaria* species found on potato in the main potato growing regions of Idaho, 39 isolates of *A. solani* were screened against four fungicides in 2009. In 2010, 9 isolates of *A. alternata* and 26 isolates of *A. solani* were screened against 12 fungicides (Table 1). The only *Alternaria* species found on potato in Idaho were *A. solani* and *A. alternata*. For most isolates, the EC_{50} was outside the range of the spiral plate dilution series (507 ppm–0.87 ppm), i.e. isolates were either insensitive or sensitive, with only a few exceptions. Isolates varied widely in their susceptibility to the fungicides tested (Tables 2 and 3).

3.2. Location is an important determinant of fungicide resistance in A. solani

Results from the disease survey of isolates collected in Idaho showed that *A. solani* was the dominant pathogen causing typical early blight symptoms in Idaho. In all the locations surveyed in southern Idaho from Parma in the southwest to Rupert to Aberdeen in the southeast, all of the diseased plants collected had typical early blight disease symptoms (irregular to circular dark brown spots up to 10 mm in diameter and restricted by veins) and none had typical brown leaf spot symptoms (small, irregular to circular, dark brown spots ranging in size from a pinpoint to 4 mm (Supplementary Fig. 2). Typical brown leaf spot symptoms were only observed on potatoes growing in Bonners Ferry, ID. These observations were confirmed by pathogen isolations from diseased

Table 2
Sensitivity of *Alternaria solani* isolates collected in 2009 from potato fields in Idaho. Isolates were tested against fungicides commonly used to control early blight in Idaho.

Idaho locations	Isolate		Fungicide sensitivity[a]			
	No.	ID	Pyraclostrobin	Boscalid	Famoxadone + cymoxanil	Azoxystrobin
Aberdeen	1	104-1	S	–[b]	R	R
Aberdeen	2	111-6	S	S	R	R
Aberdeen	3	111-7	S	–	R	R
Aberdeen	4	117-6	–	–	R	R
Aberdeen	5	117-8	S	S	S	R
Aberdeen	6	119-2	S	–	R	R
Aberdeen	7	119-3	S	–	R	R
Aberdeen	8	119-5	S	S	R	R
Aberdeen	9	119-7	S	–	R	R
Aberdeen	10	119-8	S	–	R	R
Aberdeen	11	120-8	S	–	S	R
Aberdeen	12	122-6	S	S	R	R
Aberdeen	13	124-8	–	–	R	R
Aberdeen	14	125-1	S	–	R	R
Aberdeen	15	127-3	S	S	–	R
Aberdeen	16	127-6	S	S	R	R
Aberdeen	17	215-4	–	S	R	R
Aberdeen	18	215-6	S	S	R	R
Aberdeen	19	222-2	S	S	R	R
Aberdeen	20	222-3	S	S	R	R
Aberdeen	21	222-8	S	S	R	R
Aberdeen	22	227-1	S	–	R	R
Aberdeen	23	227-2	–	S	R	R
Aberdeen	24	227-3	S	S	R	R
Aberdeen	25	325-4	S	S	R	R
Aberdeen	26	404-2	S	–	R	R
Aberdeen	27	404-5	S	–	R	R
Rupert	28	MB 204-6	S	R	R	R
Rupert	29	MB 204-7	S	–	R	R
Rupert	30	MH-4	S	R	R	R
Rupert	31	MI 107-1	S	S	R	R
Rupert	32	MI 107-3	S	–	S	R
Rupert	33	MI 109-6	S	–	R	R
Rupert	34	MI 109-7	S	R	–	R
Aberdeen	35	N-3	S	–	S	R
Aberdeen	36	N-4	S	–	S	R
Aberdeen	37	W-1	S	S	R	R
Aberdeen	38	W-5	S	–	R	R
Aberdeen	39	W-7	–	S	R	R

[a] Resistant isolates are marked with an R (growth was observed on media containing over 507 ppm fungicide) and sensitive isolates are marked with an S (growth was only observed on control plates).
[b] "–" Represents no data collected for the fungicide isolate combination.

Table 3
Sensitivity of *Alternaria* isolates against fungicides commonly used to control early blight in Idaho. Isolates were collected in 2010 from potato fields at various locations in Idaho.

Fungal species	Idaho locations	Isolate		Fungicide sensitivity[a]											
		No.	ID	Boscalid	Fluopyram	Penthiopyrad	Fluopyram + pyrimethanil	Trifloxystrobin	Pyraclostrobin	Fenamidone	Picoxystrobin	Famoxadone + cymoxanil	Azoxystrobin	Pyrimethanil	Chlorothalonil
Alternaria alternata	Bonners Ferry	101	AA1	S	S	–[c]	S	S	S	R	R	–	–	S	S
	Bonners Ferry	102	AA2	R	S	S	S	–	–	R	R	R	R	S	–
	Bonners Ferry	103	AA3	S	S	–	2.7	S	S	R	S	–	–	24	S
	Bonners Ferry	104	AA4	S	S	–	S	R	S	S	R	–	–	S	S
	Bonners Ferry	105	AA5	R	S	–	S	R	R	R	R	–	–	S	R
	Bonners Ferry	106	AA6	R	S	–	S	R	S	R	R	–	–	S	R
	Bonners Ferry	107	AA7	R	S	S	S	–	–	R	R	R	R	S	–
	Bonners Ferry	108	AA8	S	S	S	S	–	–	S	S	R	R	S	–
	Bonners Ferry	109	AA9	R	S	S	S	–	–	R	R	S	S	S	–
Alternaria solani	Rupert	110	AS1	R	S	S	S	R	R	R	R	R	R	S	R
	Rupert	111	AS2	S	S	S	S	R	S	R	R	S	R	S	S
	Rupert	112	AS3	S	S	S	S	R	S	R	R	R	R	S	R
	Rupert	113	AS4	R	S	R	S	S	S	S	S	R	R	S	S
	Rupert	114	AS5	R	S	R	S	S	S	2.7	S	R	R	S	S
	Parma	115	AS6	S	S	–	S	–	–	–	–	–	–	–	–
	Parma	116	AS7	R	39.4[b]	–	S	–	–	–	–	–	–	–	–
	Parma	117	AS8	R	S	–	S	–	–	–	–	–	–	–	–
	Parma	118	AS9	R	S	–	S	–	–	–	–	–	–	–	–
	Parma	119	AS10	R	S	–	S	–	–	–	–	–	–	–	–
	Bonners Ferry	120	AS11	S	S	2.7	S	–	–	S	S	R	R	S	–
	Aberdeen	121	AS12	S	S	S	S	R	S	R	S	R	R	S	S
	Aberdeen	122	AS13	R	S	S	S	R	S	R	R	R	R	S	R
	Aberdeen	123	AS14	R	S	S	S	S	S	R	R	S	R	S	S
	Aberdeen	124	AS15	S	S	S	S	R	S	S	S	R	R	S	S
	Aberdeen	125	AS16	R	S	S	S	R	1.6	R	R	S	R	S	S
	Aberdeen	126	AS17	R	4.8	S	S	R	R	R	R	R	R	S	R
	Aberdeen	127	AS18	R	S	S	111.9	S	S	R	R	R	R	65.5	R
	Aberdeen	128	AS19	S	S	S	S	S	S	R	R	S	R	S	S
	Parma	129	AS20	S	S	S	S	R	S	R	R	R	R	S	R
	Aberdeen	130	AS21	R	S	S	S	R	R	R	R	R	R	S	R
	Bonners Ferry	131	AS22	R	S	–	S	R	S	S	S	–	–	R	S
	Bonners Ferry	132	AS23	R	S	–	S	R	S	S	R	–	–	R	S
	Bonners Ferry	133	AS24	S	S	–	S	S	S	R	S	–	–	S	S
	Bonners Ferry	134	AS25	S	S	–	S	S	S	R	S	–	–	S	S
	Bonners Ferry	135	AS26	S	S	–	S	S	S	S	S	–	–	R	S

[a] Resistant isolates are marked with an R (growth was observed on media containing over 507 ppm fungicide) and sensitive isolates are marked with an S (growth was only observed on control plates).
[b] Isolates that have a half maximal effective concentration value (EC_{50}) have the value listed instead of R or S. EC_{50} values are denoted in ppm. EC_{50} values were rarely observed, but in those cases were categorized as sensitive if that EC_{50} value was below 10 ppm.
[c] "–" Represents no data collected for the fungicide isolate combination.

leaves collected in the different growing regions. *Alternaria solani* was the only pathogen isolated from diseased leaves collected in southern Idaho with typical early blight symptoms. *Alternaria alternata* was the only pathogen isolated from leaves collected in Bonners Ferry displaying brown leaf spot symptoms. However, *A. solani* was also isolated from leaves collected in Bonners Ferry displaying typical early blight disease symptoms.

In 2009, isolates of *A. solani* from Aberdeen were more sensitive to boscalid than isolates from Rupert and sensitivity to pyraclostrobin, famoxadone + cymoxanil, and azoxystrobin were similar in the two locations (Table 4). For 2009, there was a significant effect of location for boscalid ($P < 0.001$).

In 2010, isolates of *A. solani* collected from Bonners Ferry were more sensitive to all fungicides except pyrimethanil, compared to the *A. solani* isolates collected elsewhere in Idaho (Tables 3 and 4). Bonners Ferry was the only site that contained both *A. alternata* and *A. solani* isolates and showed that the *A. alternata* isolates had almost equal resistance to the range of fungicides tested as the *A. solani* isolates from the southern region of Idaho. *Alternaria alternata* was sensitive to five fungicides (fluopyram, penthiopyrad, fluopyram + pyrimethanil, pyrimethanil and chlorothalonil), whereas *A. solani* was sensitive to six fungicides (fluopyram, penthiopyrad, fluopyram + pyrimethanil, pyraclostrobin, fenamidone and chlorothalonil; Table 4). For 2010, most effects of location were not significant, however there was a significant effect of location for picoxystrobin ($P = 0.099$).

In the other potato growing regions in southern Idaho that were selected (Aberdeen, Rupert, and Parma), the highest levels of *A. solani* fungicide resistance were observed to the QoI fungicides (FRAC group 11; Table 5). In 2009, 15% of *A. solani* isolates were resistant to boscalid, 0% to pyraclostrobin, 86% to famoxadone and 100% to azoxystrobin. In 2010, a 41% increase in boscalid resistance and a 20% increase in pyraclostrobin resistance was observed (Table 5). When results were compared by location, all four locations contained several *A. solani* isolates that were resistant to boscalid. In 2010, the percentage of isolates resistant to boscalid were 67% from Aberdeen, 33% from Bonners Ferry, 67% from Parma, and 60% from Rupert (Table 4). Forty percent of *A. solani* isolates from Rupert were found to be resistant to penthiopyrad. While no *A. alternata* or *A. solani* isolates were identified as fully resistant to fluopyram, two *A. solani* isolates from Aberdeen had low EC_{50} values (<10 ppm; Tables 3 and 4).

Table 4
The percentage of resistant isolates detected in fungicide sensitivity screenings of *Alternaria solani*. Isolates were collected from four locations in Idaho in 2009 and 2010.

Treatment	Percentage of resistant isolates[a]				*P* value effect of location[e]
	Aberdeen	Bonners Ferry	Parma	Rupert	
2009[b]					
Boscalid	6	–	–	50	<0.001
Pyraclostrobin	0	–	–	0	N/A
Famoxadone + cymoxanil	87	–	–	83	0.812
Azoxystrobin	100	–	–	100	N/A
2010[c]					
Boscalid	67	33	67	60	0.622
Fluopyram	0	0	17	0	0.359
Penthiopyrad	0	0[d]	0[d]	40	0.195
Fluopyram + pyrimethanil	11	0	0	0	0.622
Trifloxystrobin	67	40	100[d]	60	0.698
Pyraclostrobin	22	0	0[d]	20	0.727
Fenamidone	88	33	100[d]	60	0.144
Picoxystrobin	78	17	100[d]	60	0.099
Famoxadone + cymoxanil	67	100[d]	100[d]	80	0.835
Azoxystrobin	100	100[d]	100[d]	100	N/A
Pyrimethanil	11	50	0[d]	0	0.150
Chlorothalonil	44	0	100[d]	40	0.196

[a] Half maximal effective concentration values (EC_{50}) were rarely observed, but in those cases were categorized as sensitive if that EC_{50} value was below 10 ppm. Each isolate was screened using three replicates and experiments were repeated three times.
[b] A total of 32 isolates were tested from Aberdeen and seven from Rupert.
[c] A total of nine isolates were tested from Aberdeen, six from Bonners Ferry, six from Parma and five from Rupert.
[d] Only one isolate was tested against this fungicide.
[e] *P* value calculated in JMP using output from a one-way analysis of variance for each fungicide by location.

Table 5
Fungicide sensitivity screening with isolates of *Alternaria solani* and *Alternaria alternata* collected in Idaho during 2009 and 2010.

Treatment	Percentage of resistant isolates		
	A. solani – 2009[a]	*A. alternata* – 2010[b]	*A. solani* – 2010[c]
Boscalid	15	56	58
Fluopyram	–	0	4
Penthiopyrad	–	0	13
Fluopyram + pyrimethanil	–	0	4
Trifloxystrobin	–	60	60
Pyraclostrobin	0	20	15
Picoxystrobin	–	78	57
Famoxadone + cymoxanil	86	75	75
Azoxystrobin	100	75	100
Pyrimethanil	–	11	19
Chlorothalonil	–	40	35

[a] Combined fungicide sensitivity screening results from all *A. solani* isolates collected from the two test locations in Idaho (Aberdeen and Rupert) in 2009.
[b] Combined fungicide sensitivity screening results from all *A. alternata* isolates collected from Bonners Ferry in 2010.
[c] Combined fungicide sensitivity screening results from all *A. solani* isolates collected from the four locations (Aberdeen, Bonners Ferry, Parma, and Rupert) in 2010.

3.3. Fungicide class is an important factor in Alternaria fungicide resistance

Alternaria solani isolates collected in Idaho were found to be most resistant to the fungicide azoxystrobin (FRAC group 11) with all showing resistance (Table 5) in 2009 and 2010. The isolates tested against the QoI fungicides showed the most overall growth compared to the control (denoted by black columns in Fig. 2). The fungicides in the SDH inhibitor class (FRAC group 7) that contained fluopyram had the least amount of resistant isolates, each having not a single fully resistant isolate (Table 5). However, it was noted that two isolates had low EC_{50} values in 2010 (Table 3), which made its overall growth against the controls in the midrange of fungicides tested (Fig. 2). Statistical analysis of a subset of isolates from 2010 showed significant effects for 'Isolate', 'Fungicide', and 'Isolate × Fungicide' (Table 6). In order to display statistical difference in the fungicides, means and standard errors are presented in graphical form in Fig. 2. Due to the fact that the subset mostly contained isolates from Rupert and Aberdeen, we chose to not display the interaction between isolates and fungicides.

Chlorothalonil in the multi-site contact activity class (FRAC group M5) was consistently a midrange fungicide with 35–40% of isolates being resistant (Table 5) and had 42% growth compared to the control (Fig. 2). Pyrimethanil in the methionine biosynthesis class (FRAC group 9) showed some interesting results. Alone it was found to have a few resistant *A. alternata* (11%) and *A. solani* (19%) isolates in 2010 (Table 5). However, when looking at the overall growth of the selected isolates from the most resistant areas,

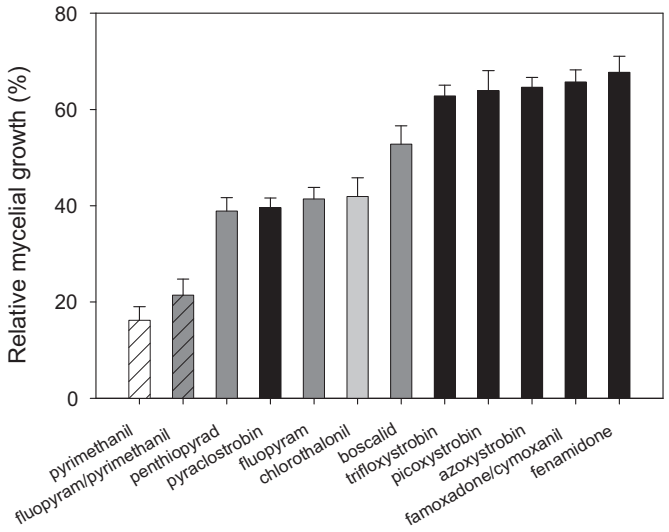

Fig. 2. Average relative growth for a subset of *Alternaria solani* isolates tested for all 12 fungicides in a sensitivity screening for resistance. A multifactor ANOVA was conducted on the subset (isolates AS1–5, and AS12–21) and significant effects were observed for isolate ($P < 0.0001$) and fungicide ($P < 0.0001$) and the interaction ($P < 0.0001$). Bars represent the standard error of the means, and different letters denote statistical significance. Note that the average relative growth for fluopyram is inflated because the growth of one isolate was promoted at low concentrations of the fungicide ($EC_{50} = 4.8$ ppm) and did not display a resistance phenotype.

(Rupert and Aberdeen) isolates on pyrimethanil amended media displayed the least amount of growth compared to the controls at 18%, with fluopyram + pyrimethanil also showing very low growth compared to the controls at 21% (Fig. 2).

4. Discussion

The results of this study strongly suggest that fungicide resistance to all the commonly used fungicides in Idaho is widespread in populations of *A. solani* and *A. alternata*. Additionally, site location and fungicide exposure appears to have an effect on the level of resistance. Specifically, isolates of both *Alternaria* species were the most resistant to the QoIs (FRAC group 11) and least resistant to inhibitors of succinate dehydrogenase (FRAC group 7) and methionine biosynthesis (FRAC group 9). Some resistant isolates were identified against particular fungicide groups that have not previously been reported in Idaho potato growing regions (e.g. boscalid and penthiopyrad). We found evidence that *A. alternata* was one third more resistant to the fungicide groups tested than *A. solani*. This could be a result of the documented widespread nature of *A. alternata*, its ability to easily sporulate and/or its saprophytic alternate lifestyle (Rotem, 1994). Further collection of additional isolates of *A. alternata* from multiple locations in Idaho is needed to confirm its greater resistance to the fungicides tested. However, this has posed a problem as in the past four years *A. alternata* isolates causing disease on potatoes have only been found in Bonners Ferry in northern Idaho.

Table 6
Summary of the analysis of variance of the main effects and interaction of isolate and fungicide in a subset of *Alternaria solani* isolates from the fungicide screening study carried out in 2010 in Idaho.

Source[a]	*n*	*F* ratio	MSE[b]	*P* value
Isolate	14	48.4	83.6	<0.001
Fungicide	11	167.6	83.6	<0.001
Isolate × fungicide	154	10.2	83.6	<0.001

[a] Significance indicated by $P < 0.05$.
[b] MSE denotes the mean standard error.

For most isolates, the EC_{50} was outside the range of the spiral plate dilution series (507 ppm–0 ppm), i.e. isolates were either completely insensitive or completely sensitive to boscalid. However, a few of the *Alternaria* isolates did have EC_{50} values. Most of the isolates that had EC_{50} values were *A. solani*, with only one isolate being *A. alternata*.

On a molecular level, single mutations or insertion sequences in promoter regions in fungicide target genes can cause resistance to develop. Resistance to the fungicide benomyl has been reported in *B. cinerea* and it has been associated with mutations in two amino acid positions of beta tubulin (Yarden, and Katan, 1993). QoIs also have a similar amino acid change that has been widely reported in many plant pathosystems. For example, *Venturia inaequalis* on apples, *Blumeria graminis* on grasses, *Mycosphaerella fijiensis* on bananas, and *Plasmopara viticola* on grapes (Gisi et al., 2000). In all of the aforementioned pathogens a mutation (G143A) causing a single amino acid change at position 143 from glycine to alanine was responsible for fungicide resistance. However, another mutation (F129L) at position 129 from phenylalanine to leucine caused reduced QoI sensitivity to develop in *A. solani* (Pasche and Gudmestad, 2008). Pasche et al. (2005) did not find full resistance to QoI fungicides in any *A. solani* isolates that they tested. They only found isolates with significantly reduced sensitivity and suggested that was a result of the fact that reduced sensitivity in *A. solani* was caused by the F129L mutation. In contrast, our results showed high levels of complete resistance to the QoI fungicides in approximately 69% of all of the *A. solani* isolates that we tested. This suggests that the F129L mutation may not be responsible for fungicide resistance in *A. solani* isolates collected in Idaho.

In an initial survey in 2009, we found 15% of *A. solani* isolates were resistant to boscalid (Wharton et al., 2012). Subsequently, a more detailed screening of *A. solani* isolates collected during 2010 showed that, depending on location, 33 to 67 percent of isolates were resistant to boscalid. These data suggest that resistance to boscalid is widespread in Idaho and increasing, even in the northern areas like Bonners Ferry where potato cultivation is extremely limited.

Five site specific mutations in succinate dehydrogenase (SDH) subunits B, C, and D have been identified for boscalid resistant isolates of *A. alternata* (Avenot et al., 2008, 2009). Boscalid resistance has also been characterized in *B. cinerea* on apples and two mutations have been identified in SDH subunit B at the amino acid positions 225 and 277 that correlate with the resistant isolates (Yin et al., 2011). Furthermore, resistance has been characterized in *Corynespora cassiicola* on cucumber and several mutations in SDH subunits B, C, and D were associated with resistance [B-H278Y, B-H278R, C-S73P, D-S89P and D-G109V (Miyamoto et al., 2010).

Even though several *Alternaria* isolates were found to be resistant to a broad range of fungicide chemistries, only two isolates developed resistance to fungicides containing only fluopyram. From our results fluopyram and penthiopyrad had significantly fewer resistant isolates than boscalid. It has been suggested by Bayer CropScience, the makers of fluopyram, that this is due to the long, linear, fluorinated molecular structure of fluopyram as compared to the densely contorted, non-fluorinated molecular structure of boscalid (Fig. 1). This may enable the binding sites of fluopyram and penthiopyrad to be more available to inhibit succinate dehydrogenase (complex II) and makes them more able to out compete ubiquinone for the ubiquinone pocket compared to its boscalid counterpart (Avenot et al., 2008). It must be noted that several boscalid resistant isolates from Rupert, Idaho showed cross resistance to penthiopyrad, but none showed resistance to fluopyram.

All isolates tested were sensitive to the fungicide mix of fluopryam + pyrimethanil (Luna Tranquility©; Bayer CropScience). This suggests that using fungicide pre-mixes may be ideal for optimal control of the pathogen and may reduce the future risk of resistance developing. The mix of fluopyram + pyrimethanil was extremely effective against all the *Alternaria* isolates and was more effective than when the two active ingredients were tested separately. During the growth assays the fluopyram + pyrimethanil mix had absolutely no growth after 4 days, whereas there was a slight amount of growth on plates when the products were tested individually. However, this growth was limited to the area which was streaked using the plastic pellet pestle and was probably the result of a small amount of fungicide amended media being removed in the process of spreading out the spore suspension with the pellet pestle (Supplemental Figs. 4 and 5). This suggests that there is some synergy between the two fungicide molecules and might also suggest a fungitoxic mode of action as opposed to a fungistatic one. Further testing is needed to determine which active ingredient in this mix is the most active, and more studies are required to understand whether there are multiple mechanisms inhibiting fungal growth.

Nine *Alternaria* isolates were found to be resistant to chlorothalonil. This was very surprising because to our knowledge, resistance to chlorothalonil has never been reported in *A. solani* previously (FRAC Code List, 2011). However, some resistance has been reported in *Phytophthora infestans* (Sujkowski et al., 1995). Chlorothalonil competes against glyceraldehyde 3-phosphate for the active site of glyceraldehyde-3-phosphate dehydrogenase and reduces fungal intracellular glutathione molecules to alternate forms (Tillman et al., 1973). It has been noted that chlorothalonil needs light to become active in controlling its target pathogens because light causes chlorothalonil to degrade into byproducts that bind specifically to the target sites (Khan and Akhtar, 1983). After application of spore suspensions to fungicide-amended plates, these plates were stored in the dark for four days. Thus, the results that were obtained may be an artifact of the incubation process. However, when we tested these isolates on poisoned PDA plates with 10,000 ppm of chlorothalonil (both the Bravo© [Syngenta Inc., NC] and Echo© [Sipcam Agro USA Inc., NC] formulations) growth was still apparent in four of the nine resistant isolates, even though the cultures were incubated in daylight for 21 days (data not shown).

The discovery of *Alternaria* isolates resistant to boscalid and penthiopyrad suggests that fungicides containing these products should be considered at high risk of resistance development. To date, boscalid and penthiopyrad insensitivity *in vitro* has not translated directly to commercial production. Fungicide spray trials carried out at the University of Idaho Aberdeen Research and Extension Center in Aberdeen, ID, show Endura© (the commercial formulation of boscalid; BASF Inc.) and Vertisan© (the commercial formulation of penthiopyrad; DuPont Crop Protection) to still be two of the most effective fungicides for controlling early blight and white mold. However, over the past two growing seasons (2011 and 2012) there have been increasing reports of Endura having a reduced efficacy against early blight in the Rupert area of southern Idaho (Jeff Miller, Miller Research, Rupert, ID, personal communication). Additionally, during the 2012 growing season, Endura performed poorly against early blight in spray trials conducted at the Aberdeen Research and Extension Center compared to its performance in previous years (results not shown). The results from this study demonstrate that growers need to be aware of the importance of rotation strategies that discourage the selection for resistance to boscalid, penthiopyrad and other related chemistries, which are currently in development.

Future work will entail more sampling of *Alternaria* populations at an increased number of locations in Idaho and at additional stages of disease development during the growing season. Data from multiple years is needed to accurately estimate the current frequency and future potential of fungicide resistance in Idaho. These data so far indicate that certain fungicide chemistries are likely more effective at controlling early blight due to the development of fungicide resistance in *Alternaria* populations. In the future this research will help growers in selecting effective fungicide chemistries to control early blight of potatoes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cropro.2013.03.003.

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