Plant Pathology (2014) **63**, 155–164 Doi: 10.1111/ppa.12077

Screening and characterization of resistance to succinate dehydrogenase inhibitors in *Alternaria solani*

T. D. Miles, L. A. Miles, K. L. Fairchild and P. S. Wharton*

Department of Plant, Soil and Entomological Sciences, University of Idaho, 1693 S 2700 W, Aberdeen, ID, 83210, USA

Field isolates of *Alternaria solani*, which causes early blight of potato in Idaho, USA were evaluated *in vitro* for their sensitivity towards the succinate dehydrogenase inhibitor (SDHI) fungicides boscalid, fluopyram and penthiopyrad. A total of 20 isolates were collected from foliar-infected tissue in 2009, 26 in 2010 and 49 in 2011. Fungicide sensitivity was tested using the spiral-gradient end point dilution method. The frequency of boscalid-resistant isolates (>50% relative growth when using a spiral dilution gradient starting at 507 mg L^{-1}) drastically increased over the duration of this study (15% in 2009, 62% in 2010 and 80% in 2011). Increasing resistance to fluopyram and penthiopyrad was observed. However, cross-resistance was only observed between boscalid and penthiopyrad. The target site of this fungicide class is the succinate dehydrogenase (SDH) enzyme complex, which is vital for fungal respiration. Sequence analysis of the SDH complex revealed mutations in the subunits B and D that were correlated with the emergence of boscalid resistance in potato fields in Idaho. In particular, H277R and H133R were identified in SDH subunits B and D, respectively. The presence of restriction sites in the gene sequences allowed the development of a rapid PCR-RFLP method to assess boscalid sensitivity in *A. solani* populations.

Keywords: boscalid, chemical control, early blight, fluopyram, penthiopyrad, *Solanum tuberosum*

Introduction

Early blight, caused by *Alternaria solani*, is one of the most devastating foliar diseases of potatoes and can reduce tuber yields by up to 60% (MacKenzie, 1981). Fortunately, a wide variety of fungicides has been developed to control early blight. These fungicides are grouped into different classes by the Fungicide Resistance Action Committee (FRAC) according to their mode of action. They include quinone outside inhibitors (QoIs, FRAC group 11), succinate dehydrogenase inhibitors (SDHIs, FRAC group 7), anilino-pyrimidines (FRAC group 9) and chloronitriles (FRAC group M5; Fungicide Resistance Action Committee, 2011). QoI fungicides were first registered for agricultural use in the USA in 1996 and are still widely used on numerous crops because of their broad-spectrum activity against ascomycetes, basidiomycetes, deuteromycetes and oomycetes (Bartlett *et al.*, 2002). However, development of resistance has become widespread throughout many potato-growing regions in the USA (Pasche & Gudmestad, 2008; Rosenzweig *et al.*, 2008a,b; Belcher *et al.*, 2010; Fairchild *et al.*, 2013).

The SDHI boscalid was released for use on potato in 2003 (Kissling, 2003) and was readily adopted by many growers in Idaho as an alternative to QoI fungicides for the control of early blight. However, in the past 3 years, Idaho potato growers have observed a failure of boscalid to control early blight in their fields (J. Miller, Miller Research, Rupert, ID, USA, personal communication). A recent study reported the development of boscalid resistance in *A. solani* isolates collected in Idaho (Wharton *et al.*, 2012).

In other pathosystems, boscalid resistance has been reported in field isolates of *A. alternata* on pistachio (Avenot & Michailides, 2007), *Botrytis cinerea* on apples and strawberries (Veloukas *et al.*, 2011; Yin *et al.*, 2011), and *Corynespora cassiicola*, *Didymella bryoniae* and *Podosphaera xanthii* on cucurbits (Miyamoto *et al.*, 2009, 2010a,b; Avenot *et al.*, 2012). Boscalid and other SDHI fungicides in FRAC group 7 target the succinate dehydrogenase complex, which blocks the citric acid cycle and the production of ATP required for fungal respiration (Avenot & Michailides, 2010). The succinate dehydrogenase (SDH) complex consists of a flavoprotein (SDH subunit A), an iron–sulphur protein (SDH subunit B) and two membrane-anchoring proteins (SDH subunits C and D). Succinate dehydrogenase inhibitors bind to a highly conserved active site composed of residues found in subunits B, C and D, where ubiquinone (coenzyme Q_{10}) is reduced to ubiquinol (Horsefield *et al.*, 2006). In general, the resistant fungal phenotypes have been attributed to the occurrence of single-site mutations in subunits B, C and D of the SDH complex (Avenot & Michailides, 2010). In the closely related species *A. alternata*, which causes late blight of pistachio, boscalid resistance has

*E-mail: pwharton@uidaho.edu

Published online 22 May 2013

been associated with two single-nucleotide mutations in SDH subunit B (H277Y and H277R; Avenot *et al.*, 2008) and mutations in the membrane-anchoring subunits C (H134R) and D (H133R and D123E; Avenot *et al.*, 2009). In isolates of *B. cinerea* on apples, two mutations in SDH subunit B, at amino acid positions 225 and 277, correlated with boscalid resistance (Yin *et al.*, 2011). Furthermore, in *C. cassiicola* on cucumber, several mutations in SDH subunits B, C, and D were associated with boscalid resistance (B-H278Y, B-H278R, C-S73P, D-S89P and D-G109V; Miyamoto *et al.*, 2010a).

Recently, the Environmental Protection Agency (EPA) in the USA has registered several new fungicides containing SDHI active ingredients; penthiopyrad (Fontelis, Vertisan, DuPont Crop Protection) and fluopyram (Luna, Luna Tranquility, Bayer CropScience). To date, fungicide resistance has not been observed for fluopyram. However, there is some evidence of cross-resistance in boscalid-resistant isolates of *D. bryoniae*, *C. cassiicola* and *P. xanthii* to penthiopyrad (Ishii *et al.*, 2011; Avenot *et al.*, 2012).

The aim of this research was to survey and characterize fungicide sensitivity in populations of *A. solani* in Idaho to the commonly used SDHIs boscalid, fluopyram and penthiopyrad. This research also aimed to develop a molecular test to rapidly screen and specifically detect resistant isolates of this important pathogen of potato.

Materials and methods

Collection and isolation of *A. solani* single-spore isolates

Potato leaves with lesions characteristic of early blight were collected from potato fields in Aberdeen, Bonners Ferry, Parma and Rupert, ID, USA. Fields had been treated with at least four applications of fungicides per growing season starting when the potato crop had reached row closure (plant canopies touching between rows), and included multisite fungicides (e.g. chlorothalonil), QoIs and SDHIs. Leaf samples were transported on ice to the laboratory in plastic resealable bags and pressed between filter papers to dry. To obtain isolates, a small piece of dried tissue (5 × 5 mm) was excised from the centre of a lesion and streaked across the surface of a thin layer (3 mm) of tap water agar (TWA). After incubation overnight at 25°C, single germinated spores were transferred aseptically to acidified (0·05% [v/v] acetic acid at 0·5 ml L^{-1}) potato dextrose agar (APDA; Difco) and incubated at 25°C in the dark for 5–7 days.

Identification and maintenance of *A. solani* isolates

For species identification, isolates were transferred onto potato dextrose agar (PDA; Difco) and incubated at 25°C in the dark for at least 3 weeks, with 5-min periods of sunlight exposure every week to increase sporulation (Rotem, 1994). *Alternaria solani* isolates were identified based on morphological traits (Simmons, 2007). Additionally, identification to the species level was confirmed by sequence analysis of the internal transcribed spacer regions (ITS1 and ITS2) of nuclear ribosomal DNA, using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) from White *et al.* (1990) and the following PCR conditions: an initial denaturation step at 94°C for 2 min, followed by 40 amplification cycles (denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min) and a final elongation step at 72°C for 10 min. The products were sequenced in both directions and sequences were assembled to form a contiguous sequence using Geneious Pro v. 4.7.6 software (Biomatters Ltd.) and analysed using blastn and blastx (GenBank).

A total of 20 single-spore isolates were obtained in 2009, 26 in 2010 and 49 in 2011. Every single-spore isolate was maintained at room temperature (22–24°C) on slants containing 30 mL PDA media.

SDHI fungicide sensitivity *in vitro*

All *A. solani* isolates collected between 2009 and 2011 were evaluated against commercially formulated fungicides containing boscalid (screened in 2009–2011), fluopyram (screened in 2010 and 2011) or penthiopyrad (screened in 2010 and 2011; FRAC group 7) using the spiral-gradient end point dilution method (Förster *et al.*, 2004). Four commercial fungicides were evaluated: Endura (70% active ingredient [a.i.] boscalid; BASF), Luna (42·4% a.i. fluopyram; Bayer CropScience), Luna Tranquility (a mixture of 11·3% a.i. fluopyram and 33·8% a.i. pyrimethanil; Bayer CropScience) and Vertisan (20·6% a.i. penthiopyrad; DuPont Crop Protection). Fungicide stock solutions were prepared at 10 000 mg L^{-1} in distilled water and were spiral-plated using an IUL Instruments Eddy Jet 1700 automatic spiral plater (Neutec Group Inc.) onto 15-cm PDA plates amended with rifamycin (75 mg L^{-1}) and ampicillin (200 mg L^{-1}). The concentration at which the fungicides were plated ranged between 0·87 and 507 mg L^{-1} a.i. *Alternaria solani* isolates tested previously on non-antibiotic-amended PDA plates showed no significant differences in colony growth between antibiotic-amended and non-amended plates in fungicide screening assays (data not shown).

Three-week-old sporulating cultures of *A. solani* were flooded with 5 mL sterile distilled water (SDW) and spores were then dislodged from the media using a sterile bent glass rod by gently scraping the surface of the colony. The spore suspension was then filtered through two layers of sterile cheesecloth and placed in a 50-mL sterilized beaker. Each spore suspension was measured with a haemocytometer and adjusted to a concentration of 1·0–2·0 × 10^4 spores mL^{-1}. A 10 μL aliquot of this suspension was applied as a line across the fungicide-amended plate using a micropipette. Three lines were applied per plate (Fig. S1a,c). Plates were incubated at 25°C in the dark and fungal growth was assessed 4 days after inoculation by measuring the largest diameter of the colony. At least two replicate plates were used per isolate/fungicide combination and the experiment was conducted three times. Control, non-fungicide-amended PDA plates were also included as reference of fungal colony growth (Fig. S1b,d). The effective concentration of boscalid that inhibited 50% of the fungal colony growth (EC_{50}) was estimated as described below.

Plates to be assessed were placed face down on a flat bed scanner (Epson Perfection V500 PHOTO; Epson America Inc.) and an image was captured, imported into photoshop CS5 (Adobe Inc.), 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 × 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 PDA was used in each Petri dish so that the distance between the scanner surface and medium in the plate was the same throughout all experiments. This was important to ensure that the brightness of the images was consistent throughout the experiment.

Images were analysed in PHOTOSHOP CS5 using the ruler tool. The analysis scale was set to 119 pixels, which equalled 1 cm. 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 (>507 mg L^{-1}) compared to the control plate, the isolate was considered to be resistant to the test fungicide.

To test the sensitivity of *A. solani* to boscalid, a subset of 15 *A. solani* isolates (AS1, AS3, AS4, AS5, AS6, AS8, AS9, AS11, AS13, AS15, AS17, AS18, AS20, AS23 and AS25) collected in Idaho, USA in 2010 was evaluated using the classic poisoned-agar technique. Petri dishes (10 cm diameter) containing 20 mL PDA amended with boscalid at a concentration of 0·1, 1, 10, 100 or 1000 mg L^{-1} were inoculated with a 5-mm mycelium plug of each isolate (Fig. 1). Plates were incubated at 25°C in the dark and fungal growth was assessed (i.e. measure of colony diameter in cm) 7 days after inoculation. Three replicate plates were used per isolate. Non-fungicide-amended PDA plates were included as fungal growth controls. All EC_{50} values were calculated by linear regression analysis of the growth inhibition percentage relative to the control according to the logarithm of the active ingredient concentration.

Mutations in the SDH subunits

The subset of 15 isolates were also used for molecular characterization of the succinate dehydrogenase (SDH) complex. DNA was extracted using the standard phenol:chloroform based protocol from Sambrook *et al.* (1989). Subunits B, C and D were amplified by PCR with three different primer sets (Table 1) using a PCR programme that initiated with a denaturation step at 94°C for 2 min, followed by 40 amplification cycles (denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min) and a final elongation step at 72°C for 10 min. Products were sequenced in both directions. The sequences obtained for every SDH subunit were assembled into a single contiguous sequence and analysed to identify single-site mutations using GENEIOUS (v. 4.7.6, Biomatters Ltd.).

Single nucleotide polymorphism detection by PCR-RFLP

Primers were developed to amplify two sequence fragments surrounding two predominant single-site mutations (H277R in the SDH subunit B, and H133R in the SDH subunit D) present in the SDH complex of boscalid-resistant *A. solani* isolates (Table 2). Unique restriction sites were identified, using GENEIOUS, to selectively differentiate between wildtype (sensitive) and mutant (resistant) genotypes in the PCR product. Restriction enzymes (*Aci*I, *Hpy*166II and *Bsr*BI) were purchased from New England Biolabs. PCR products (10 μL) were digested for at least 1 h in a 20-μL reaction following the manufacturer's protocols. PCR products (5 μL) and enzyme digestion products (10 μL) were loaded and visualized on a 2% agarose gel in 1 × TBE with ethidium bromide.

Results

In vitro sensitivity to SDHI fungicides

In most cases, the calculation of an EC_{50} value was encumbered by the fact that the value lay outside the range of the spiral plate dilution series (0·87–507 mg L^{-1}). In order to facilitate a phenotypic assignment (i.e. in terms of fungicide sensitivity), isolates that were unable to grow or grew up to 50% on fungicide-amended plates were classified as 'sensitive' (0–50% growth relative to the control; Fig. S1a) and those isolates that grew at >50–100% growth relative to the control (Fig. S1c) were classified as 'resistant' using a spiral dilution gradient starting at 507 mg L^{-1}.

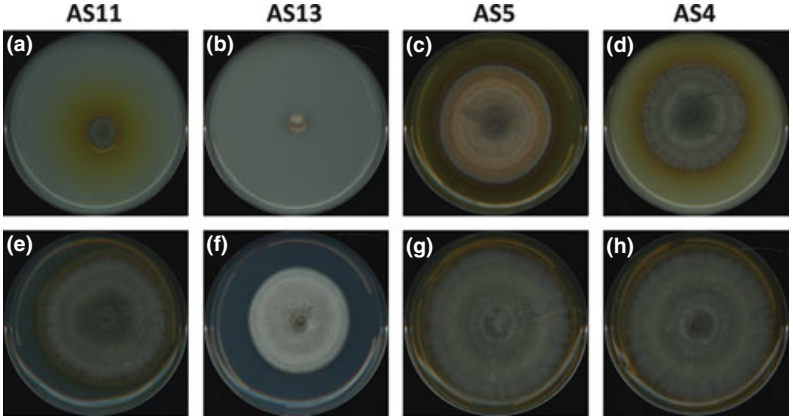

Figure 1 Poisoned-agar technique to evaluate boscalid sensitivity of four *Alternaria solani* isolates from Idaho, USA potato fields in 2010. (a–d) PDA plates amended with 1000 mg L^{-1} boscalid; (e–h) non-amended PDA control plates. The observable growth of isolates AS5 and AS4 on the boscalid-amended plates demonstrates *in vitro* resistance.

Table 1 Primer sets used for the amplification and sequencing of the succinate dehydrogenase (SDH) subunits B, C and D in *Alternaria solani*

Primer	Primer sequence (5′–3′)	Source	Tm (°C)[a]	Amplified DNA fragment[b]
SDH subunit B				
SDHB-2F	ATA CGC GCT TTC ACT CGT CT	This study	60·0	10 to 1020
SDHB-2R	GCA TGT CCT TGA GCA GTT GA	This study	60·0	
SDHB-F2	GTG GCG TCG AAG GGC CGA AGA AGC CG	Avenot *et al.* (2008)	68·9	−33 to 349
SDHB-R2	CAG CAT CAT GGG TCC GGT CTT GTT GA	Avenot *et al.* (2008)	62·7	
SDHB-F3	TAC TGG TGG AAC CAG GAG GAG TA	Avenot *et al.* (2008)	58·8	856 to 1130
SDHB-R3	CAT ACC ACT CTA GGT GAA G	Avenot *et al.* (2008)	49·0	
SDH subunit C				
SDHC-1F	GCT TCT CAG CGG GTA TTT CA	This study	60·4	4 to 600
SDHC-2R	CTG AAT GCG ACG GTC AAG	This study	58·9	
SDHC-F2	ATG GCT TCT CAG CGG GTA TTT CAG	Avenot *et al.* (2009)	59·1	1 to 284
SDHC-R2	GAA GGT GTA GTA AAG GCT GCA GG	Avenot *et al.* (2009)	57·6	
SDHC-F3	CCA AAT CAC CTG GTA CGC CTC G	Avenot *et al.* (2009)	60·2	284 to 623
SDHC-R3	TCA TCC GAG GAA GGT GTA GTA AAG GCT G	Avenot *et al.* (2009)	61·2	
SDH subunit D				
SDHD-2F	CCG GTC TCC TCA GGC AAG	This study	62·0	20 to 613
SDHD-1R	CTA TGC GTG CCA CAA CCT C	This study	60·3	
SDHD-F2	GTT CGT CGT TCC GAT ATT GCA TC	Avenot *et al.* (2009)	56·2	−23 to 306
SDHD-R2	AGA GAT GAT GCG CTC AAA ACT CCA	Avenot *et al.* (2009)	58·6	
SDHD-F3	CGC CGG TCC CGA ACC CAG ACT ACT	Avenot *et al.* (2009)	66·3	464 to 634
SDHD-R3	CAG CCA AAG GAC ACA AAC TCG	Avenot *et al.* (2009)	56·6	

[a]Predicted primer melting temperature.
[b]Fragment numbering corresponds to nucleotide positions in the SDH subunits B, C and D of *A. solani*, with the first nucleotide of the start codon considered as position +1.

Table 2 Primer sets and restriction enzymes used for determining the presence of mutations in *Alternaria solani* succinate dehydrogenase (SDH)

Mutation	Primer	Primer sequence (5′–3′)	Tm (°C)[a]	Amplicon size (bp)	Restriction enzyme assay
H277R (SDH subunit B)	F-H277R-B	TGC CCA TCG TAC TGG TGG AAC C	61·5	168	Digest with *Acl*I or *Fnu*4HI. No cleavage of amplicon indicates histidine at codon 277; digestion products of 33 and 135 bp indicates arginine.
	R-H277R-B	GGG GCA TGT CCT TGA GCA GTT GA	61·7		
H133R (SDH subunit D)	F-H133R-D	CCG ACT CTA TTC TCT GCG CCC T	60·7	167	Digest with *Hpy*166II or *Bsr*BI. *Hpy*166II digestion products of 32 and 135 bp indicates histidine at codon 133; *Bsr*BI digestion products of 34 and 133 bp indicates arginine.
	R-H133R-D	CTC GAA AGA GTA GAG GGC AAG ACC CA	61·5		

[a]Predicted primer melting temperature.

The percentage of isolates with a SDHI-fungicide resistant phenotype revealed a trend towards increased resistance to boscalid among the *A. solani* isolates collected in potato fields in Idaho from 2009 to 2011 (Table 3). Similar trends were also observed for fluopyram and penthiopyrad resistance but further testing is required to see if this trend will continue in successive years (Table 3). While an overall reduction in growth was observed on fluopyram-amended plates in 2011, a small percentage of isolates (9%, 4 of 46 isolates; Table 3) were classified as resistant. However, the isolates were only able to grow slightly above a 50% level relative to the growth of the control when using a spiral dilution gradient starting at 507 mg L^{-1}. In the case of penthiopyrad, a significant increase in resistance was observed in 2011 as compared to 2010 (Table 3). Development of resistance to a mixture of fluopyram and pyrimethanil (Luna Tranquility) was not observed in this study.

Results demonstrated cross-resistance between boscalid and penthiopyrad in *A. solani* isolates but showed no cross-resistance between boscalid and fluopyram. In 2010, two of the penthiopyrad-resistant isolates were also resistant to boscalid. However in 2011, 31 penthiopyrad-resistant isolates were detected: 26 were also resistant to boscalid, but five showed sensitivity to boscalid (two with a calculated EC_{50} value; Table 4). For fluopyram, all 2010 isolates were sensitive; in 2011, four isolates were resistant but only three of these were boscalid-resistant (Table 4).

Table 3 The percentage of resistant and sensitive *Alternaria solani* isolates collected from potato fields in Idaho, USA from 2009 to 2011 against four succinate dehydrogenase inhibitor fungicide formulations[a]

Year of collection	Fungicide	Trade name	No. of isolates tested	Resistant isolates (%)[b]
2009	Boscalid	Endura	20	15
2010	Boscalid	Endura	26	62
	Fluopyram	Luna	26	0
	Fluopyram + pyrimethanil	Luna Tranquility	26	0
	Penthiopyrad	Vertisan	16	13
2011	Boscalid	Endura	46	80
	Fluopyram	Luna	46	9
	Fluopyram + pyrimethanil	Luna Tranquility	46	0
	Penthiopyrad	Vertisan	49	55

[a]Each isolate was screened using at least two replicates and experiments were conducted three times.
[b]A resistant isolate was defined as an isolate with 50% or more colony growth relative to the control using a spiral dilution gradient starting at 507 mg L^{-1}. EC_{50} values could rarely be calculated, but in those cases isolates were categorized as sensitive.

Characterization of *in vitro* boscalid resistance

Fifteen *A. solani* isolates collected in Idaho during 2010 were further evaluated with a poisoned-agar technique in order to calculate an accurate EC_{50} value for each isolate (Fig. 1). The results revealed a wide range of growth, which suggested a wide range of sensitivity to boscalid, with EC_{50} values ranging from 5·8 to >1000 mg L^{-1} (Table 5). Isolates AS11, AS13, AS15 and AS20 were classified as sensitive with the spiral-plate dilution technique. For these isolates, the relative growth ranged between 30·8 and 44·2% when using a spiral dilution gradient starting at 507 mg L^{-1} and the EC_{50} values ranged from 5·8 to 12·7 mg L^{-1} (Table 5). The other evaluated isolates (AS1, AS3, AS4, AS5, AS6, AS8, AS9, AS17, AS18, AS23 and AS25) had been classified as resistant to boscalid using the spiral-plate dilution technique. They all had a relative growth higher than 50% and it was corroborated with the poisoned-agar technique (relative growth ranged from 52·5 to 89·9% when using the spiral dilution gradient starting at 507 mg L^{-1}; Table 5). However, the poisoned-agar method revealed two sensitivity groups within these resistant isolates, a group with intermediate EC_{50} values that ranged from 10·3 to 92·2 mg L^{-1}, and another group with high EC_{50} values that were >1000 mg L^{-1} (Table 5).

Single-point mutations in the SDH complex

Four nucleotide changes were discovered in the SDH subunit B and D sequences of seven of 11 isolates classified as boscalid-resistant using *in vitro* techniques. These mutations were all single-nucleotide sequence changes and included H277R in SDH subunit B and T28A, A47T

Table 4 Sensitivity to four succinate dehydrogenase inhibitor fungicide formulations[a] in *Alternaria solani* isolates collected from potato fields in Idaho, USA in 2010 and 2011 using the spiral-gradient end point dilution method

			Fungicide sensitivity (resistant, sensitive, or EC_{50} in mg L^{-1})[b]			
Year	Isolate	Field location	Bos	Flu	Flu+Pyr	Pen
2010	AS1	Rupert	R	S	S	S
	AS2	Rupert	S	S	S	S
	AS3	Rupert	R	S	S	S
	AS4	Rupert	R	S	S	R
	AS5	Rupert	R	S	S	R
	AS6	Parma	R	S	S	–
	AS7	Parma	R	39·4	S	–
	AS8	Parma	R	S	S	–
	AS9	Parma	R	S	S	–
	AS10	Parma	R	S	S	–
	AS11	Bonners Ferry	S	S	S	2·7
	AS12	Aberdeen	S	S	S	S
	AS13	Aberdeen	S	S	S	S
	AS14	Aberdeen	R	S	S	S
	AS15	Aberdeen	S	S	S	S
	AS16	Aberdeen	R	S	S	S
	AS17	Aberdeen	R	4·8	S	S
	AS18	Aberdeen	R	S	111·9	S
	AS19	Aberdeen	S	S	S	S
	AS20	Parma	S	S	S	S
	AS21	Aberdeen	R	S	S	S
	AS22	Bonners Ferry	R	S	S	–
	AS23	Bonners Ferry	R	S	S	–
	AS24	Bonners Ferry	S	S	S	–
	AS25	Bonners Ferry	R	S	S	–
	AS26	Bonners Ferry	S	S	S	–
2011	AS201	Rupert	R	S	S	R
	AS202	Rupert	R	S	S	R
	AS203	Rupert	R	S	S	R
	AS204	Rupert	R	14·9	S	R
	AS205	Rupert	R	R	S	R
	AS206	Rupert	39·4	S	S	R
	AS207	Rupert	R	2·7	S	R
	AS208	Rupert	R	14·9	S	R
	AS209	Rupert	4·8	R	S	R
	AS210	Rupert	R	S	S	R
	AS211	Rupert	R	14·9	S	R
	AS212	Rupert	R	1·6	S	R
	AS215	Rupert	R	S	S	R
	AS217	Rupert	R	4·8	S	R
	AS218	Rupert	R	S	S	R
	AS219	Rupert	R	2·7	S	R
	AS220	Rupert	R	S	S	R
	AS221	Rupert	R	1·6	S	R
	AS222	Rupert	R	S	S	R
	AS223	Rupert	R	R	S	R
	AS224	Rupert	R	0·9	S	R
	AS225	Jerome	R	S	S	R
	AS226	Jerome	R	S	S	S
	AS227	Jerome	R	1·6	S	R
	AS228	Jerome	R	1·6	S	R
	AS229	Jerome	R	4·8	S	S
	AS230	Jerome	R	1·6	S	S

(continued)

Table 4 (continued)

Year	Isolate	Field location	Fungicide sensitivity (resistant, sensitive, or EC_{50} in mg L^{-1})[b]			
			Bos	Flu	Flu+Pyr	Pen
	AS231	Jerome	S	S	S	S
	AS232	Jerome	R	0·9	1·6	R
	AS234	Aberdeen	R	S	0·9	S
	AS235	Aberdeen	R	S	S	R
	AS236	Aberdeen	–	–	–	S
	AS237	Aberdeen	–	–	–	S
	AS238	Aberdeen	–	–	–	S
	AS239	Aberdeen	2·7	2·7	S	S
	AS240	Aberdeen	R	0·9	S	R
	AS245	Bonners Ferry	S	S	S	S
	AS246	Bonners Ferry	R	2·7	S	S
	AS247	Bonners Ferry	R	S	S	S
	AS248	Bonners Ferry	R	S	S	R
	AS249	Bonners Ferry	2·7	S	S	S
	AS250BF	Bonners Ferry	R	S	S	S
	AS250P	Parma	S	0·9	S	R
	AS253	Parma	S	S	S	R
	AS254	Parma	S	S	S	R
	AS255	Parma	R	0·9	S	S
	ASS101	Aberdeen	R	S	S	S
	ASS103	Aberdeen	R	S	S	S
	ASS104	Aberdeen	R	R	S	S

[a]The fungicides evaluated were boscalid (Bos), fluopyram (Flu), fluopyram + pyrimethanil (Flu + Pyr) and penthiopyrad (Pen).
[b]R: resistant isolate, $EC_{50} > 507$ mg L^{-1}; S: sensitive isolate, $EC_{50} \leq 0{\cdot}87$ mg L^{-1}; –: no data collected.

and H133R in SDH subunit D. In most isolates, more than one mutation was detected. Interestingly, mutation T28A in SDH subunit D was always observed to coincide with mutation A47T (Table 5). However, mutation H277R in SDH subunit B was detected either on its own or along with mutation H133R in SDH subunit D (Table 5). In one instance, mutation H277R in SDH subunit B was detected when mutations T28A and A47T occurred in SDH subunit D (Table 5).

Isolates AS4 and AS5, with the highest relative growth when using a spiral dilution gradient starting at 507 mg L^{-1} (>80%) and the highest EC_{50} values (>1000 mg L^{-1}) possessed both the H277R mutation in SDH subunit B and H133R in SDH subunit D (Table 5). In this study, no nucleotide changes were observed in the SDH sequences of *A. solani* isolates classified as sensitive (Table 5). Furthermore, no nucleotide changes were observed in the sequence of SDH subunit C.

PCR-RFLP detection of H277R and H133R

The development of a PCR-RFLP assay to detect single-nucleotide changes in the sequences of SDH subunits B and D allowed the differential identification of wildtype (fungicide-sensitive isolates) and mutant (fungicide-resistant isolates) genotypes using the presence or absence of restriction sites (Fig. S2). When mutation H277R is present in the sequence of SDH subunit B, a restriction enzyme site for *Aci*I or *Fnu*4HI is generated (Fig. S2a). In the same way, for the identification of mutation H133R in the sequence of SDH subunit D, the restriction enzymes *Hpy*166II and *Bsr*BI can be used to identify the wildtype and mutant genotypes, respectively (Fig. S2b).

The RFLP analysis of SDH subunit B with the restriction enzyme *Aci*I clearly identified mutation H277R in the boscalid-resistant *A. solani* isolates AS4 and AS5 by cleaving the 168-bp DNA sequence into two fragments of size 33 and 135 bp (Fig. 2, lanes 7 and 9). In contrast, the enzyme did not digest the DNA of the boscalid-sensitive isolates AS11 and AS13 (Fig. 2, lanes 3 and 5).

For the detection of mutation H133R in SDH subunit D, two DNA fragments of 32 and 135 bp were generated when using restriction enzyme *Hpy*166II on boscalid-sensitive isolates (wildtype genotype), whilst the PCR product of isolates with the mutation remained undigested (Fig. 3, lanes 3, 5, 7, 9). Conversely, *Bsr*BI cleaved the PCR product of boscalid-resistant isolates (mutant genotype), into 34 and 133 bp fragments (Fig. 3, lanes 16 and 18), but did not cleave the DNA of sensitive-isolates (Fig. 3, lanes 12 and 14).

In order to validate the PCR-RFLP technique, one isolate from 2010 (AS20, of known SDH sequence) and 13 additional isolates from 2011 (of unknown sequence) were tested for the presence of H277R and H133R. Seven isolates (including AS4 and AS5, of known sequence) possessed the H277R mutation in SDH subunit B and all were resistant to boscalid and penthiopyrad using the spiral dilution assay described previously, whereas only five of these isolates (AS4, AS5, AS205, AS209 and AS223) possessed the H133R mutation in SDH subunit D (Table 6). However, five isolates that were resistant to both boscalid and penthiopyrad did not contain either mutation.

Discussion

Potato growers in Idaho rely mainly on fungicide applications to manage early blight (Harrison & Venette, 1970; Douglas & Groskopp, 1974). Since the rapid and widespread development of QoI resistance (Pasche & Gudmestad, 2008), growers have become increasingly reliant on other chemistries such as fungicides belonging to the SDHI class. In southern Idaho, because of dry weather conditions and minimal late blight disease pressure, growers typically make as few as three fungicide applications during a growing season, two of which would normally include SDHI fungicides.

In the present study the SDHI-fungicide sensitivity of *A. solani* isolates collected from potato fields in Idaho during three growing seasons was evaluated. The results suggest that populations of *A. solani* in Idaho, under constant selection pressure, are likely to develop resistance to SDHI fungicides over time, particularly to boscalid, because it has been used longer commercially. In addition to that, there was evidence of development of resistance towards two other SDHI fungicides,

Table 5 Nucleotide mutations and deduced alterations in the amino acid sequence of the succinate dehydrogenase (SDH) subunits B, C and D in boscalid-resistant isolates of *Alternaria solani* as compared to four sensitive isolates (AS11, AS13, AS20 and AS15) collected from four sites in Idaho, USA in 2010

Isolate		Relative		SDH subunit B	SDH subunit C	SDH subunit D			
Code	Origin	growth (%)[a]	EC_{50} (mg L^{-1})[b]	Codon 277	Codon 134	Codon 28	Codon 47	Codon 123	Codon 133
AS11	Bonners Ferry	30·8	5·8	H (CAC)[c]	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS13	Aberdeen	35·0	9·5	H (CAC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS20	Parma	40·4	7·7	H (CAC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS15	Aberdeen	44·2	12·7	H (CAC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS3	Rupert	52·5	10·3	H (CAC)	H (CAC)	**A (G**CA)	**T (A**CC)	D (GAC)	H (CAC)
AS8	Parma	53·4	21·5	H (CAC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS9	Parma	56·8	14·9	H (CAC)	H (CAC)	**A (G**CA)	**T (A**CC)	D (GAC)	H (CAC)
AS6	Parma	57·2	26·1	H (CAC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS1	Rupert	58·0	23·1	H (CAC)	H (CAC)	**A (G**CA)	**T (A**CC)	D (GAC)	H (CAC)
AS23	Bonners Ferry	58·7	29·9	H (CAC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS25	Bonners Ferry	62·7	23·4	H (CAC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS17	Aberdeen	76·4	92·2	**R (C**GC)[d]	H (CAC)	**A (G**CA)	**T (A**CC)	D (GAC)	H (CAC)
AS18	Aberdeen	77·1	46·2	**R (C**GC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	H (CAC)
AS5	Rupert	83·7	>1000	**R (C**GC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	**R (C**GC)
AS4	Rupert	89·9	>1000	**R (C**GC)	H (CAC)	T (ACA)	A (GCC)	D (GAC)	**R (C**GC)

[a]Fungal growth on boscalid-amended plates (poisoned-agar technique) relative to the control (n = 3) when using a spiral dilution gradient starting at 507 mg L^{-1}.
[b]Effective concentration of boscalid that inhibited 50% of the fungal colony growth, calculated from a poisoned-agar technique.
[c]Amino acid (codon); A: alanine; D: aspartic acid; H: histidine; R: arginine; T: threonine.
[d]Mutations, compared to isolate AS11, are denoted in bold.

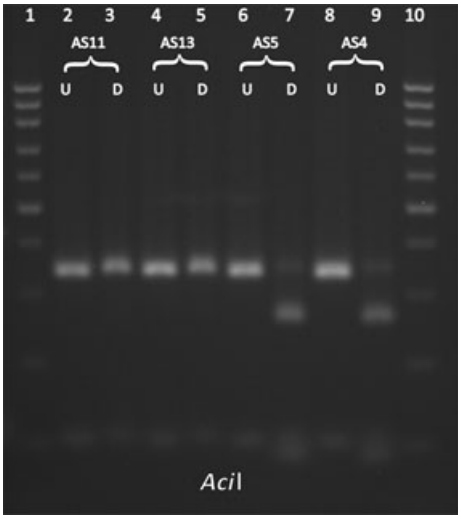

Figure 2 Detection of the mutation H277R in the succinate dehydrogenase (SDH) subunit B by PCR-RFLP. The restriction enzyme *Aci*I cleaves the 168-bp PCR amplicon (Table 2) of boscalid-resistant *Alternaria solani* isolates (AS5, AS4) into 33- and 135-bp fragments. Lanes 2, 4, 6, 8: undigested PCR products (U); lanes 3, 5, 7, 9: *Aci*I-digested PCR products (D) from AS11, AS13, AS5 and AS4, respectively. Lanes 1, 10: 50-bp ladder.

penthiopyrad and fluopyram. However, a greater insensitivity was detected to penthiopyrad than to fluopyram in the last year of this study (2011). The differences in SDHI sensitivity to the three fungicides evaluated are probably a result of entirely different fungicide chemistries. Fluopyram and penthiopyrad are fluorinated, long, linear compounds, whereas boscalid is a non-fluorinated, dense compound (Fraaije *et al.*, 2012; Musson & Young, 2012). Fluopyram might also be more effective at inhibiting *A. solani* growth *in vitro* because of the multiple molecular configurations that the compound can have. This ability might increase the competitiveness of fluopyram for the active site of the SDH complex and allow it to interact with more amino acid residues (Musson & Young, 2012). Interestingly, the highly boscalid-resistant isolates (AS4 and AS5) showed cross-resistance to penthiopyrad, whereas all of the 15 further characterized isolates were sensitive to fluopyram in the spiral-gradient end point dilution test. These results corroborate findings with *A. alternata* on pistachio (Avenot & Michailides, 2010), *C. cassiicola* and *P. xanthii* on cucumber (Ishii *et al.*, 2011) and *D. bryoniae* on watermelon (Avenot *et al.*, 2012), where a lack of cross-resistance between boscalid and penthiopyrad with fluopyram was found. This suggests that the binding site of fluopyram in the SDH complex may differ slightly from that of boscalid and penthiopyrad (Avenot & Michailides, 2010; Ishii *et al.*, 2011; Avenot *et al.*, 2012).

The identification of mutations in SDHI-resistant isolates has been reported in several pathosystems (Avenot *et al.*, 2008; Miyamoto *et al.*, 2010a; Veloukas *et al.*, 2011; Yin *et al.*, 2011; Fraaije *et al.*, 2012). In general, the most common mutation found to confer SDHI resistance was the replacement of a highly conserved histidine residue in the SDH subunit B by tyrosine or arginine (H277R/Y; Avenot & Michailides, 2010). In accordance with previous reports, the current study showed that the

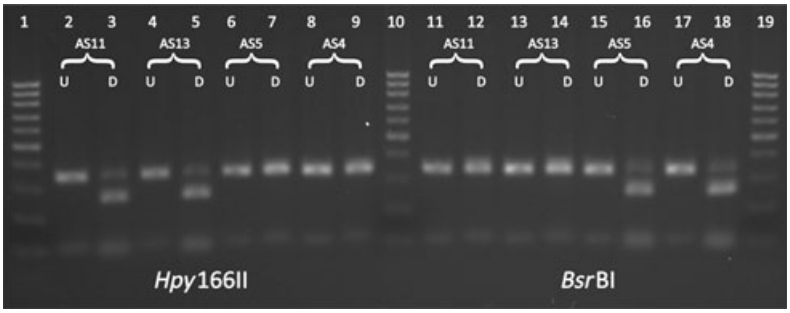

Figure 3 Detection of the wildtype genotype and the mutation H133R in the succinate dehydrogenase (SDH) subunit D by PCR-RFLP. *Hpy*166II cleaves the 167-bp PCR amplicon (Table 2) of boscalid-sensitive *Alternaria solani* isolates (AS11, AS13) into 32- and 135-bp fragments; *Bsr*BI cleaves the amplicon of boscalid-resistant isolates (AS5, AS4) into 34- and 133-bp fragments. Lanes 2, 4, 6, 8, 11, 13, 15 and 17: undigested PCR products (U); lanes 3, 5, 7, 9: *Hpy*166II-digested PCR products (D); lanes 12, 14, 16, 18: *Bsr*BI-digested PCR products (D) from AS11, AS13, AS5 and AS4, respectively. Lanes 1, 10, 19: 50-bp ladder.

Table 6 Detection of nucleotide mutations present in succinate dehydrogenase inhibitor (SDHI)-resistant isolates of *Alternaria solani* using a PCR-RFLP method to identify two amino acid substitutions in SDH subunit B (H277R) and SDH subunit D (H133R). Isolates were collected from Idaho potato fields in 2010 and 2011

	Fungicide sensitivity (resistant, sensitive, or EC_{50} in mg L^{-1})[a]			SDH subunit B H277R[b]	SDH subunit D H133R[b]
Isolate	Bos	Flu	Pen		
SDHI-sensitive isolates					
AS11	S	S	S	–	–
AS13	S	S	S	–	–
AS20	S	S	S	–	–
SDHI-resistant isolates					
AS4	R	S	R	+	+
AS5	R	S	R	+	+
AS201	R	S	R	+	–
AS202	R	S	R	–	–
AS204	R	14·9	R	+	–
AS205	R	R	R	–	+
AS207	R	2·7	R	+	–
AS208	R	14·9	R	–	–
AS209	R	R	R	–	+
AS211	R	14·9	R	–	–
AS212	R	1·6	R	+	–
AS217	R	4·8	R	+	–
AS219	R	2·7	R	–	–
AS221	R	1·6	R	–	–
AS223	R	R	R	–	+

[a]Fungicide sensitivity was evaluated *in vitro* against four SDHI fungicide formulations using the spiral-gradient end point dilution method. Bos: boscalid; Flu: fluopyram; Pen: penthiopyrad; R: resistant isolate, 50% or more colony growth relative to the control or EC_{50} > 507 mg L^{-1}; S: sensitive isolate; $EC_{50} \leq$ 0·87 mg L^{-1}.
[b]–: PCR-RFLP indicates presence of codon for histidine; +: PCR-RFLP indicates presence of codon for arginine.

mutation H277R in SDH subunit B and the mutation H133R in SDH subunit D are the most critical for the development of highly boscalid-resistant *A. solani* isolates. Furthermore, it was possible to detect isolates that possessed both mutations. These isolates were also observed to have a higher resistance phenotype than those isolates with only the H277R mutation. However, because no resistant *A. solani* isolates with only the mutation H133R were detected, it is not possible to determine if the H133R mutation confers higher resistance. In the closely related species *A. alternata*, the occurrence of multiple mutations in individual isolates was not identified (Avenot *et al.*, 2008, 2009). However, *in vitro* studies of *Mycosphaerella graminicola* showed that SDHI-resistant isolates with multiple mutations in the SDH complex were more sensitive to SDHIs than those carrying single nucleotide substitutions. Fitness costs related to these mutations were not detected (Fraaije *et al.*, 2012).

The mutations T28A and A47T in SDH subunit D have not been reported previously. These single nucleotide changes were always found together and occurred in four of nine moderately resistant isolates (boscalid EC_{50} values of 10·3–92·2 mg L^{-1}). However, the EC_{50} values overlapped slightly with sensitive isolates (5·8–12·7 mg L^{-1}). Further investigation is required to determine if T28A and A47T are involved in boscalid resistance or are a natural variant. Moreover, mutations in the SDH subunit C were not found, whilst 52·6% of the boscalid-resistant *A. alternata* isolates from pistachio had mutations in this subunit (Avenot *et al.*, 2009; Avenot & Michailides, 2010). This suggests that in *A. alternata* there is a higher mutation frequency in SDH subunit C than in subunit B (39% of the isolates) and subunit D (7·9% of the isolates; Avenot *et al.*, 2008, 2009; Avenot & Michailides, 2010).

The use of molecular detection technologies to differentiate resistant and sensitive isolates will improve the ability to rapidly assess SDHI sensitivity in the field. However, prior characterization of the pathogen population under study is essential to associate the predominant mutations to a specific level of SDHI sensitivity. The PCR-RFLP method developed in this work is capable of specifically detecting the two predominant mutations that were identified in *A. solani* populations in Idaho. This

method is a two-step process after DNA extraction: amplification of the specific sequence from the SDH subunit B or D, followed by the digestion of the specific single-nucleotide polymorphism (SNP). The only shortcoming with the method is the inability to detect mutations in five SDHI resistant isolates. This failure to detect the presence of the H277R and H133R mutations in these isolates leads to the hypothesis that other SNPs, such as H277Y, might be present and confer SDHI resistance (Avenot *et al.*, 2008). Similar PCR-RFLP methods have been proposed for use to detect mutations in *A. alternata* on pistachio (Avenot *et al.*, 2008, 2009) and in *B. cinerea* on strawberries (Veloukas *et al.*, 2011). Methods that have been implemented for the detection of QoI resistance could also be adapted for detecting SNPs that confer SDHI resistance. These alternative methods include technologies such as a Q-PCR-hybridization melt-curve assay (Pasche *et al.*, 2005), a TaqMan SNP genotyping assay (Samuel *et al.*, 2011), or an amplification refractory mutation system (ARMS)-SYBR Green Q-PCR assay (Baudoin *et al.*, 2008). However, these molecular tools require a higher initial investment and additional optimization steps to develop. Because SDHI resistance has been linked to several SNPs in the SDH complex, characterizing a population and all of the associated mutations to resistance in a single assay would be unachievable. However, the implementation of several similar assays would ensure the rapid detection of the most prevalent mutations.

Acknowledgements

Funding was provided in part by Bayer CropScience. Fungicides were provided by Bayer CropScience, BASF Inc., and DuPont Crop Protection.

References

Avenot HF, Michailides TJ, 2007. Resistance to boscalid fungicide in *Alternaria alternata* isolates from pistachio in California. *Plant Disease* **91**, 1345–50.

Avenot HF, Michailides TJ, 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection* **29**, 643–51.

Avenot HF, Sellam A, Karaoglanidis G, Michailides TJ, 2008. Characterization of mutations in the iron–sulphur subunit of succinate dehydrogenase correlating with boscalid resistance in *Alternaria alternata* from California pistachio. *Plant Disease* **98**, 736–42.

Avenot HF, Sellam A, Michailides T, 2009. Characterization of mutations in the membrane-anchored subunits *AaSDHC* and *AaSDHD* of succinate dehydrogenase from *Alternaria alternata* isolates conferring field resistance to the fungicide boscalid. *Plant Pathology* **58**, 1134–43.

Avenot HF, Thomas A, Gitaitis RD, Langston DB, Stevenson KL, 2012. Molecular characterization of boscalid- and penthiopyrad-resistant isolates of *Didymella bryoniae* and assessment of their sensitivity to fluopyram. *Pest Management Science* **68**, 645–51.

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B, 2002. The strobilurin fungicides. *Pest Management Science* **58**, 649–62.

Baudoin A, Olaya G, Delmotte F, Colcol JF, Sierotzki H, 2008. QoI resistance of *Plasmopara viticola* and *Erysiphe necator* in the mid-Atlantic United States. *Plant Health Progress*. doi: 10.1094/PHP-2008-0211-02-RS.

Belcher AR, Wood E, Wharton PS, 2010. Sensitivity of *Alternaria solani* populations in Idaho to commonly used fungicides and its effect on potato early blight management in Idaho. *Phytopathology* **100**, S14.

Douglas DR, Groskopp MD, 1974. Control of early blight in eastern and southcentral Idaho. *American Potato Journal* **51**, 361–8.

Fairchild KL, Miles TD, Wharton PS, 2013. Assessing fungicide resistance in populations of *Alternaria* in Idaho potato fields. *Crop Protection* **49**, 31–9.

Förster H, Kanetis L, Adaskaveg JE, 2004. Spiral gradient dilution, a rapid method for determining growth responses and 50% effective concentration values in fungus–fungicide interactions. *Phytopathology* **94**, 163–70.

Fraaije BA, Bayon C, Atkins S, Cools HJ, Lucas JA, Fraaije MW, 2012. Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. *Molecular Plant Pathology* **13**, 263–75.

Fungicide Resistance Action Committee, 2011. FRAC Code List: Fungicides sorted by mode of action (including FRAC code numbering). CropLife International, Brussels, Belgium. [http://www.frac.info]. Accessed 4 December 2012.

Harrison MD, Venette JR, 1970. Chemical control of potato early blight and its effect on potato yield. *American Potato Journal* **47**, 81–6.

Horsefield R, Yankovskaya V, Sexton G *et al.*, 2006. Structural and computational analysis of the quinone-binding site of complex II (succinate-ubiquinone oxidoreductase): a mechanism of electron transfer and proton conduction during ubiquinone reduction. *Journal of Biological Chemistry* **281**, 7309–16.

Ishii H, Miyamoto T, Ushio S, Kakishima M, 2011. Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Corynespora cassiicola* and *Podosphaera xanthii*. *Pest Management Science* **67**, 474–82.

Kissling E, 2003. *EPA Registers Endura and Pristine Fungicides. BASF News Release*. Ludwigshafen, Germany: BASF SE.

MacKenzie DR, 1981. Association of potato early blight, nitrogen fertilizer rate, and potato yield. *Plant Disease* **65**, 575–7.

Miyamoto T, Ishii H, Seko T, Kobori S, Tomita Y, 2009. Occurrence of *Corynespora cassiicola* isolates resistant to boscalid on cucumber in Ibaraki Prefecture, Japan. *Plant Pathology* **58**, 1144–51.

Miyamoto T, Ishii H, Stammler G *et al.*, 2010a. Distribution and molecular characterization of *Corynespora cassiicola* isolates resistant to boscalid. *Plant Pathology* **59**, 873–81.

Miyamoto T, Ishii H, Tomita Y, 2010b. Occurrence of boscalid resistance in cucumber powdery mildew in Japan and molecular characterization of the iron-sulfur protein of succinate dehydrogenase of the causal fungus. *Journal of General Plant Pathology* **76**, 261–7.

Musson G, Young H, 2012. Status of fluopyram resistance development. *Phytopathology* **102** (Suppl. 4), S4.84.

Pasche JS, Gudmestad NC, 2008. Prevalence, competitive fitness and impact of the F129L mutation in *Alternaria solani* from the United States. *Crop Protection* **27**, 427–35.

Pasche JS, Piche LM, Gudmestad NC, 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Disease* **89**, 269–78.

Rosenzweig N, Atallah ZK, Olaya G, Stevenson WR, 2008a. Evaluation of QoI fungicide application strategies for managing fungicide resistance and potato early blight epidemics in Wisconsin. *Plant Disease* **92**, 561–8.

Rosenzweig N, Olaya G, Atallah ZK, Cleere S, Stanger C, Stevenson WR, 2008b. Monitoring and tracking changes in sensitivity to azoxystrobin fungicide in *Alternaria solani* in Wisconsin. *Plant Disease* **92**, 555–60.

Rotem J, 1994. *The Genus* Alternaria: *Biology, Epidemiology, and Pathogenicity*. St Paul, MN, USA: American Phytopathological Society.

Sambrook J, Fritsch EF, Maniatis T, 1989. *Molecular Cloning: A Laboratory Manual*. 2nd edition. New York, USA: Cold Spring Harbor Laboratory Press.

Samuel S, Papayiannis LC, Leroch M, Veloukas T, Hahn M, Karaoglanidis GS, 2011. Evaluation of the incidence of the G143A mutation and *cytb* intron presence in the *cytochrome bc-1* gene conferring QoI resistance in *Botrytis cinerea* populations from several hosts. *Pest Management Science* **67**, 1029–36.

Simmons EG, 2007. Alternaria, *An Identification Manual*. CBS Biodiversity Series No. 6. Utrecht, The Netherlands: CBS Fungal Biodiversity Center.

Veloukas T, Leoroch M, Hahn M, Karaoglanidis GS, 2011. Detection and molecular characterization of boscalid-resistant *Botrytis cinerea* isolates from strawberry. *Plant Disease* **95**, 1302–7.

Wharton P, Fairchild K, Belcher A, Wood E, 2012. First report of *in-vitro* boscalid-resistant isolates of *Alternaria solani* causing early blight of potato in Idaho. *Plant Disease* **96**, 454.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York, USA: Academic Press Inc., 315–22.

Yin YN, Kim YK, Xiao CL, 2011. Molecular characterization of boscalid resistance in field isolates of *Botrytis cinerea* from apple. *Phytopathology* **101**, 986–95.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. *Alternaria solani* isolates grown on spiral-gradient end point dilution plates. (a) The boscalid sensitive isolate AS11 plated out on a boscalid-amended plate; (b) AS11 growing on a boscalid-free control plate (white arrows). Note there is very limited spore germination in the area where the spore suspension was applied to the surface of the medium in (a). (c) The boscalid resistant isolate AS4 growing on a boscalid-amended plate; (d) AS4 growing on a boscalid-free control plate (white arrows). Boscalid concentrations on the fungicide-amended plates ranged from 507 mg L^{-1} in the centre to 0·87 mg L^{-1} at the edge of the plates.

Figure S2. (a) Alignment of succinate dehydrogenase (SDH) subunit B gene sequences from a boscalid sensitive (AS11, wild type, WT) and a boscalid resistant (AS4) *Alternaria solani* isolate, indicating the differential restriction site for H277R. (b) Alignment of SDH subunit D gene sequences, indicating the differential restriction sites for H133R.