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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.

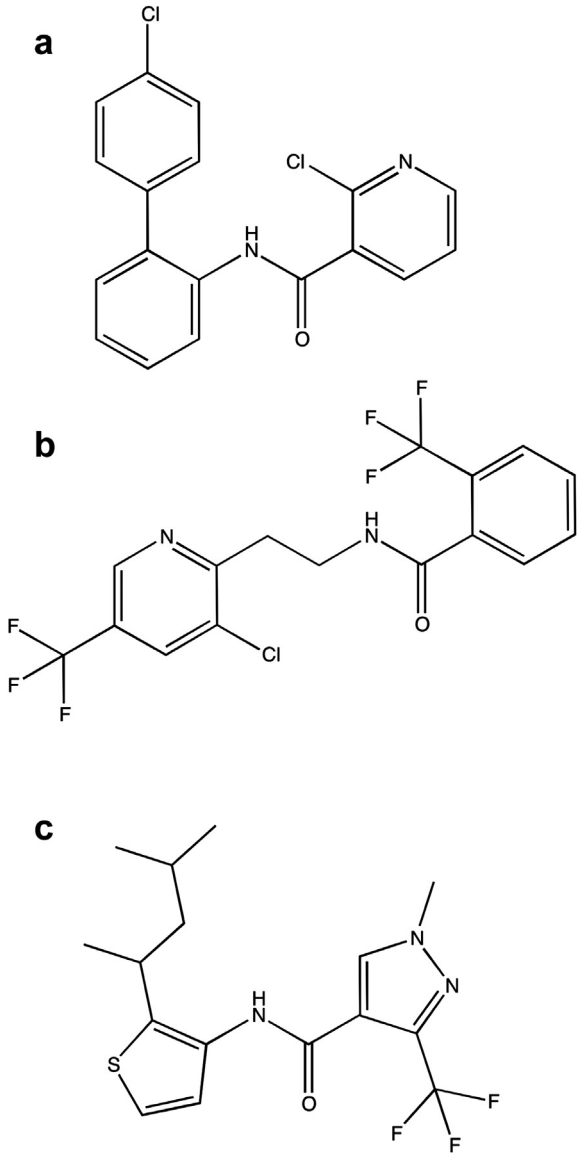

Fig. 1. Chemical structures of succinate dehydrogenase inhibitor fungicides (FRAC group 7) including: a) boscalid (2-chloro-*N*-(4′-chlorobiphenyl-2-yl)nicotinamide) b) fluopyram (*N*-{2-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethyl}-α,α,α-trifluoro-*o*-toluamide) and c) penthiopyrad ((*RS*)-*N*-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)pyraz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