



Boscalid-resistance in *Alternaria alternata* and *Alternaria solani* populations: An emerging problem in Europe



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ABSTRACT

The application of fungicides is the most common and most effective method to control *Alternaria* diseases in potatoes. Boscalid, a member of the succinate dehydrogenase inhibiting (SDHI) fungicide group, is frequently used in combination with Quinone outside inhibitors (QoIs) such as strobilurins in spraying programs. However, due to the frequent application of these fungicides, resistant populations can develop. Indeed, resistance towards SDHIs is an emerging problem in both *A. alternata* and *A. solani* populations. To gain deeper knowledge on the prevalence of SDHI mutants, 83 *A. solani* and 53 *A. alternata* isolates, collected during 2014 and 2015 in Belgium, situated in the center of Europe, were screened for the presence of amino acid substitutions in the different subunits of the succinate dehydrogenase gene (*SdhB*, *SdhC* and *SdhD*). The isolate screening revealed that mutations, causing a reduced sensitivity towards SDHIs, were widespread in the Belgian *Alternaria* population; 70% of the *A. solani* and 41% of the *A. alternata* isolates possessed one or more mutations. Mutations in the subunit *SdhC* were most frequently detected in the *A. solani* population, while mutations in the subunit *SdhD* were absent. In the *A. alternata* population, mutations were found in the three *Sdh* subunits. Mutations in the subunit *SdhB* were predominant, whereas mutations in the subunit *SdhD* were only marginally detected. Furthermore, it was seen that most isolates with a QoI mutation, providing resistance towards strobilurins, simultaneously carried substitutions in the *Sdh* gene. This means that 40% of the *A. solani* and 58% of the *A. alternata* population possesses a dual fungicide resistance. This is very important since SDHIs and QoIs are often formulated together in one fungicide. The fungicide sensitivity tests confirmed that the isolates with mutations were significantly less sensitive towards the SDHI boscalid compared to the isolates with neither a SDHI nor a QoI mutation. No significant differences in resistance level between isolates with mutations in the different subunits were detected. Furthermore, based on mycelium growth and spore germination capacity, there was no fitness cost associated with these mutations.

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1. Introduction

Alternaria leaf blight is a highly destructive disease of potatoes worldwide, leading to significant yield losses. The closely related

species *Alternaria solani* and *Alternaria alternata* are the main pathogens found in *Alternaria* lesions on potato leaves (Van der Waals et al., 2001; Leiminger and Hausladen, 2012). Both pathogens differ in some morphological features such as mycelium growth rate, spore structure and temperature requirements (Kapsa and Osowski, 2007). In general, *A. alternata* is considered a weak pathogen with an opportunistic lifestyle, whereas *A. solani* is considered an aggressive species (Rotem, 1994; Turkensteen et al., 2010; Spoelder et al., 2014; Stammler et al., 2014). The characteristic symptoms of *Alternaria* infections on leaves are dark brown to black spots with concentric rings, often surrounded by a yellow

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halo (Rotem, 1994).

Due to its polycyclic nature and the capacity of *Alternaria* species to produce high amounts of secondary inoculum *Alternaria* leaf blight is difficult to manage (Campo et al., 2007). Cultural practices such as crop rotation, weed management, irrigation and adequate fertilization can help to reduce the impact of the disease. However, in most cases chemical control is essential to avoid significant yield losses. To effectively control the disease, multiple fungicide applications during the growing season are necessary (Rosenzweig et al., 2008; Horsfield et al., 2010).

Respiration inhibitors, such as quinone outside inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs), play a key role in the management of *Alternaria* diseases. QoIs inhibit the electron transport chain in the mitochondrial respiratory chain of the complex bc1, also known as complex III (Bartlett et al., 2002), while SDHIs interfere with the enzyme succinate dehydrogenase (*Sdh*), a component of complex II in the mitochondrial electron transport chain (Kuhn, 1984). Despite having a similar biological mode of action, SDHI fungicides show no cross-resistance to QoI fungicides. This makes them excellent candidates for alternating with, or mixing with, QoI fungicides to manage fungicide resistance development while also providing superior disease control (Avenot et al., 2008a). Nevertheless, due to the single-site mode of action of both QoIs and SDHIs, there is a medium to high risk for resistance development towards these fungicides (<http://www.frac.info/working-group/>).

The QoI fungicide azoxystrobin was introduced for use on potato in 1999 in the US and provided an excellent control of *Alternaria* species. However, in recent years a reduction in fungicide sensitivity has been reported. This can be attributed to point mutations in the mitochondrial target gene, *cytochrome b* (*cyt b*). The G143A mutation (i.e., in position 143, a glycine (G) is replaced by an alanine (A)), conveys absolute resistance in various pathogens such as *A. alternata* (Grasso et al., 2006). In *A. solani*, only the F129L mutation (i.e., the substitution of phenylalanine (F) by leucine (L) at position 129) has been observed, which results in a reduced sensitivity towards QoI fungicides (Pasche et al., 2004; Leiminger et al., 2014).

The boscalid-based fungicides Endura[®] (70% boscalid) and Pristine[®] (25.2% boscalid and 12.8% pyraclostrobin) were registered in the US in 2003. Unfortunately, a reduced sensitivity towards boscalid has been reported in 2005 in *A. alternata* isolates from Pistachio in California. The majority of the isolates from orchards without a prior history of boscalid usage had ED50-values ranging from 0.089 to 3.435 µg/mL, whereas in orchards with a history of boscalid usage, highly resistant *A. alternata* isolates, with ED50-values exceeding 100 µg/mL, were found (Avenot and Michailides, 2007).

In 2009 in Idaho, the first resistance in *A. solani* towards boscalid was detected (Wharton et al., 2012). Also in other parts of the US (North Dakota, Minnesota, Nebraska and Texas) a reduced sensitivity of *A. solani* isolates towards boscalid has been reported (Gudmestad et al., 2013). Approximately 80% of all *A. solani* isolates assayed were found to have some level of resistance to boscalid. A moderate level of resistance appeared in 5% of the isolates, with ED50-values ranging from 5 to 20 µg/mL. High levels of resistance, up to 500 µg/mL boscalid, were observed in the remaining 75% of the isolates. Interestingly, 99% of all boscalid resistant isolates possessed the F129L mutation in the *cyt b* gene, indicating that an *A. solani* population with dual fungicide resistance predominates in the states surveyed.

In recent studies, the genetic characterization of the succinate dehydrogenase gene (*Sdh*) complex was performed (Avenot et al., 2014; Mallik et al., 2014; Miles et al., 2014). As mentioned above, the target site of SDHIs is the mitochondrial *Sdh* complex. This

complex consists of four subunits: flavoprotein (Fp or *SdhA*), iron–sulfur protein (Ip or *SdhB*) and two membrane-anchored proteins (*SdhC* and *SdhD*) (Hagerhall, 1997). A reduced susceptibility towards SDHI fungicides has been attributed to several point mutations in the subunits: *SdhB*, *SdhC* and *SdhD* of the *Sdh* gene complex. In both *A. alternata* and *A. solani*, sequencing of the subunit *SdhB* elucidated a point mutation cytosine (C) to thymine (T) at nucleotide position 990, leading to an amino acid exchange from histidine (H) to tryptophan (Y) at amino acid position 278 (H278Y) for *A. solani* and at position 277 (H277Y) for *A. alternata*. Another mutation from adenine (A) to guanine (G) at nucleotide position 991 of the subunit *SdhB* conferred an amino acid exchange from H to arginine (R) at amino acid position 278 (H278R) for *A. solani* and at position 277 (H277R) for *A. alternata* (Avenot et al., 2008b; Mallik et al., 2014). In the subunit *SdhC* a mutation from A to G at nucleotide position 490 was observed, leading to a H134R exchange. A similar mutation was found in the subunit *SdhD* at position 398 resulting in a H133R mutation. Furthermore, also the D123E mutation, due to a point mutation from a C to A at nucleotide position 369 in the subunit *SdhD* has been detected (Avenot et al., 2009; Mallik et al., 2014).

Avenot et al. (2014) showed that *A. alternata* isolates with a reduced sensitivity towards boscalid mainly harbor amino acid substitutions in subunits *SdhB* (H277Y and H277R) and *SdhC* (H134R), with the H277Y mutation being predominant. Only one isolate had the H133R change in the subunit *SdhD*. Furthermore, two mutants carried both the H277Y (*SdhB*) and H134R (*SdhC*) mutations.

In *A. solani* isolates collected from potato fields in the US (Colorado, Idaho, Minnesota, Nebraska, North Dakota, and Texas) during 2011, the five different described mutants were present (Mallik et al., 2014). The H278Y and H278R mutations were the predominant, being detected with a frequency of respectively 51% and 23%. Eight percent of the mutant isolates carried the *SdhC* H134R mutation. The H133R mutation in the subunit *SdhD* was present in 16% of the isolates, whereas the D123E mutation was rarely detected (2%). In the study of Miles et al. (2014) only the *SdhB* H278R mutation and the *SdhD* H133R mutations were detected in the *A. solani* population of Idaho. Furthermore, the mutation H278R in *Sdh* subunit B was detected either on its own or along with mutation H133R in the *Sdh* subunit D.

Cross-resistance patterns across SDHI fungicides are complex due to the fact that some mutations confer high levels of resistance and others do not and mutations could lead to different sensitivity shifts in different SDHIs (Olaya et al., 2016). Avenot et al. (2014) investigated the cross-resistance patterns between different SDHI fungicides in the *A. alternata* from pistachio orchards in California. Some *SdhB* or *SdhC* mutants displayed highly sensitive, sensitive, or weakly sensitive phenotypes towards penhiopyrad or fluxapyroxad, which are in the same cross-resistance group as boscalid, whereas other had low, moderate, or high levels of resistance to these fungicides. In contrast, all the *SdhB* mutants were sensitive to fluopyram, while some *SdhC* mutants had sensitive, weakly sensitive, and moderately resistant fluopyram phenotypes, respectively. The *SdhD* mutant had reduced sensitivity to fluopyram and penhiopyrad, but was highly resistant to fluxapyroxad. The discrepancies of the cross-resistance patterns between SDHIs suggest that their binding sites in complex II may differ slightly and that additional mechanisms of resistance to these compounds are likely involved.

Since *Alternaria* leaf blight on potatoes is an emerging problem in North West Europe, it is important to map the prevalence of SDHI mutations and to gain insight into the sensitivity of the *Alternaria* isolates towards the frequently used SDHI fungicide: boscalid. Therefore, a genetic characterization of the *Sdh* subunits of *A. solani* and *A. alternata* isolates collected in Belgium was performed.

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