

Fungicide Resistance in Bavarian *Alternaria solani* and *Alternaria alternata* Field Isolates

MATHIAS NOTTENSTEINER¹, CAROLIN ABSMEIER², MICHAEL ZELLNER³

¹ Bavarian State Research Center for Agriculture, Institute für Plant Protection, Lange Point 10, 85354 Freising, Germany

² Bavarian State Research Center for Agriculture, Institute für Plant Protection, Lange Point 10, 85354 Freising, Germany

³ Bavarian State Research Center for Agriculture, Institute für Plant Protection, Lange Point 10, 85354 Freising, Germany

SUMMARY

Alternaria leaf spots caused by *Alternaria solani* and *Alternaria alternata* are a major foliar disease of potatoes. Reduced sensitivity of *A. solani* and *A. alternata* towards Quinone outside inhibitors (QoI) fungicides has been observed in Europe. QoI-insensitive *A. alternata* isolates carry a G143A amino acid exchange caused by a single nucleotide polymorphism (SNP) in the cytochrome *b* gene. *A. solani* evolved a similar F129L mutation. A shift from the predominant *A. solani* genotype I to genotype II, which was exclusively associated with the F129L mutation, was observed in Germany after QoI approval for Alternaria control. Here, we found QoI mutations to be highly abundant in *A. solani* and *A. alternata* field isolates, collected in 2016 in Bavaria, located in southeastern Germany. We observed an increase in the frequency of the F129L mutation but not of *A. solani* genotype II. Instead, we also found the F129L mutation in genotype I, indicating QoI resistance progression through the previously unaffected genotype. In contrast to previous data, QoI mutations were present in all examined areas, indicating a spatial spread. An analysis of SNP diversity pointed to the region of Lower Bavaria as a hotspot for F129L mutation evolution and rather to its dispersal than to multiple independent evolutions. Reduced sensitivity of *Alternaria* spp. towards succinate dehydrogenase inhibitors (SDHI) fungicides is an emerging problem in Europe. We observed the presence of SDHI mutations, but only in combination with QoI mutations, indicating a further adaption to applied fungicides and a selection for dual fungicide resistance.

KEYWORDS

Alternaria alternata, *Alternaria solani*, Fungicide Resistance, Quinone outside Inhibitors (QoI), Succinate Dehydrogenase Inhibitors (SDHI), potatoes

INTRODUCTION

Parasitic species of the Ascomycota genus *Alternaria* cause numerous of plant diseases on diverse crop plants throughout the world (Rotem 1994). *Alternaria solani* Sorauer and *Alternaria alternata* (Fr.) Keissl. are the major *Alternaria* spp. with the potential of causing economic important yield losses of potato (*Solanum tuberosum* L.) (Leiminger and Hausladen 2012). Foliar diseases caused by *A. solani* and *A. alternata* on potato are Early Blight and Brown Spot, respectively. Symptoms of Early Blight are necrotic spots on the leaf surface that typically contain dark concentric rings (bullseye or target spot symptom). The spots may be surrounded by a chlorotic halo. They initially appear a few mm in size before they enlarge to up to 2 cm. They are limited by the leaf veins. First symptoms are observable on older leaves close to the soil surface. Overwintering inoculum in the soil or on plant debris is considered as the source of primary infection. Later in the season, together with the maturation of leaves, Early Blight spots progress upwards. Older spots enlarge, may converge and get brittle. Elongated spots may additionally appear patchy on petioles and stems. Sporulation on the lesions and subsequent wind dispersal of numerous conidia cause secondary infections on the plant, in the field and in neighboring fields. Heavily diseased leaves become premature senescent and necrotic. The reduced photosynthetic capacity of damaged leaves and the premature defoliation of plants can cause yield losses of up to 20% which are rarely exceeded. Brown Spot symptoms resemble Early Blight spots but are smaller in size, darker in color and appear more frequent and occasionally earlier in the season. Large amounts of inoculum on or in the soil can cause tuber lesions in storage when the tubers were wounded at harvesting. (Radtke et al. 2000; Stevenson et al. 2001) The symptoms of Early Blight and Brown Spot may get confused and both pathogens can co-occur in the same lesions. In Germany, *A. solani* is recognized as the dominant pathogen causing yield losses of potato, whereas *A. alternata* is considered a secondary pathogen with a more opportunistic lifestyle (Rotem 1994; Leiminger et al. 2015).

In parts of the world, Early Blight has been a major potato disease for decades and centuries. Reports on problems with Early Blight in the Midwest of the USA date back to 1893 (Pscheidt and Stevenson 1986). In Australia, Early Blight can be of greater importance than Late Blight caused by *Phytophthora infestans* (Dillard et al. 1993). In Europe, although having been latent present, Early Blight of potato has not been in the focus of plant protection until reports on an increase in the frequency and severeness of the disease in the field a decade ago (Leiminger 2009). Early Blight was considered as an emerging disease in Europe and has been included as an option in chemical plant protection strategies against Late Blight. Currently especially very susceptible as well as late season varieties might require additional *Alternaria* spp. control.

A range of fungicide active ingredients registered for Late Blight control also contain a good efficacy for *Alternaria* spp. control, e.g. the multi-site inhibitors mancozeb and the related metiram or the Quinone outside inhibitor (QoI) famoxadone (LFL 2018). If those (and others not mentioned) are not used in Late Blight control and if further factors, like heat and drought stress, additionally increase the conduciveness to Early Blight disease, then additional applications of fungicides with a greater efficacy in *Alternaria* spp. control can be of an economic advantage in southern Germany. Current fungicide products registered for *Alternaria* spp. control in potatoes in Germany contain azoxystrobin, pyraclostrobin, boscalid and difenoconazole as antifungal agents and mixtures of them or mixtures with active ingredients against *P. infestans*. Azoxystrobin and pyraclostrobin are QoI (FRAC code 11) with a high risk for fungicide resistance, known resistance in various fungal species and cross resistance between all QoI. Boscalid is a succinate dehydrogenase inhibitor (SDHI) (FRAC code 7) with a medium to high risk for

fungicide resistance and known resistance in several fungal species. Difenoconazole is a triazole of the demethylation inhibitor (DMI) (FRAC code 3) group with a medium risk for fungicide resistance. (FRAC 2018a) Due to observed degradation of sensitivity of some fungal species to sterol biosynthesis inhibitors (SBI) (Price *et al.* 2015), it is recommended not to use one single SBI for the control of one pathogen in multiple applications (FRAC 2018b) as required in the case of *Alternaria* spp. control. For the sake of keeping *Alternaria* spp. populations as long as possible sensitive to antifungal agents, fungicide resistance management practices are of utmost importance.

QoI are single-site respiration inhibitors. Biochemically, they act by binding to the Qo site of cytochrome b of the cytochrome bc₁ complex (complex III) at the inner mitochondrial membrane thereby inhibiting the electron transport and subsequent ATP production (Bartlett *et al.* 2002). Azoxystrobin was registered for Early Blight control in 1999 in the USA and found to be highly effective in the American Midwest, where Early Blight is notoriously difficult to control. A loss of sensitivity of collected *A. solani* field isolates to azoxystrobin was already observed in the year 2000 in lab trials, with a further increase of the EC₅₀ values in the year 2001. Notably, this shift in sensitivity was also observed in isolates collected from fields that have not been treated with azoxystrobin. By 2006, 96.5% of U.S. *A. solani* isolates, also collected from potato growing areas outside the Midwest, had a reduced sensitivity to azoxystrobin. The rapid loss of sensitivity towards azoxystrobin was likely due to multiple applications of azoxystrobin as single compound in Early Blight control during the growing seasons (Pasche and Gudmestad 2008).

The loss of sensitivity to QoI fungicides is mainly attributed to single nucleotide polymorphisms (SNPs) in the cytochrome b (*cytb*) gene of phytopathogenic fungi leading to amino acid substitutions in the Qo pocket of the cytochrome b protein that abolish QoI 'ligand' binding (Bartlett *et al.* 2002; Gisi *et al.* 2002). The most prominent amino acid substitution is from glycine to alanine at position 143 (G143A). The G143A substitution typically causes strong to complete loss of sensitivity towards QoI fungicides (Gisi *et al.* 2000) and cross-resistance within QoI fungicides (Pasche and Gudmestad 2008). Sequencing of the *cytb* gene of diverse phytopathogenic fungi revealed the presence of an intron directly after the codon coding for glycine at amino acid position 143 in some of the species, which is absent in species showing the G143A substitution. There, a SNP changing the codon to code for alanine would prevent splicing of the intron that would likely be lethal. Therefore, species with such a gene structure are unlikely to evolve the G143A substitution. However, they are able to gain the F129L mutation, where phenylalanine is replaced by leucine at amino acid position 129 in the Qo pocket of cytochrome b (Grasso *et al.* 2006). The F129L substitution was found to cause a more differential loss in sensitivity to QoI. The effect of the F129L substitution reduces the sensitivity towards QoI but does not result in complete resistance and cross-resistance within QoI fungicides (Kim *et al.* 2003; Pasche *et al.* 2005).

A. alternata can gain the G143A substitution, whereas *A. solani* cannot, but can instead gain the F129L substitution (Grasso *et al.* 2006). U.S. *A. solani* F129L mutant isolates were 10-15-fold less sensitive to azoxystrobin in spore germination assays compared to wildtype isolates and showed a robust cross-resistance to pyraclostrobin. However, there was just a subtle cross-resistance with famoxadone, which is a QoI but not a strobilurine. Please note that famoxadone is generally less effective than azoxystrobin in sensitive *A. solani* populations. (Pasche *et al.* 2004; Pasche *et al.* 2005; Pasche and Gudmestad 2008) This loss of sensitivity appears modest when e.g. compared to the 200-fold decrease of sensitivity towards QoI caused by the G143A

substitution in *Blumeria graminis* f.sp. *tritici* (Sierotzky *et al.* 2000). However, the F129L substitution reduced the efficacy of azoxystrobin in Early Blight control in field trials to the level of mancozeb, such that azoxystrobin has lost its superiority in Early Blight control in the USA (Pasche and Gudmestad 2008).

In Germany, azoxystrobin and pyraclostrobin plus boscalid were registered for *Alternaria* spp. control in potatoes in 2007 and 2008, respectively, due to a preceding increase of *Alternaria* diseases of potato (Leiminger and Hausladen 2012). The leaf blight caused by *Alternaria* spp. is only hardly attributable to *A. solani* or *A. alternata* by eye in the field and both pathogens can co-occur on diseased fields (Hausladen and Leiminger 2007). Species-specific real-time PCR pointed to *A. solani* being the more abundant species, although that was not entirely consistent between years (Leiminger *et al.* 2015). In 2009, first F129L substitutions were detected at a very low frequency (5%) in *A. solani* isolates collected from potato fields in Lower Bavaria in southern Germany. The frequency of *A. solani* F129L mutant isolates rapidly rose to 74% until 2011 in Bavaria although the distribution of the F129L substitution remained patchy in and between potato growing areas. (Leiminger *et al.* 2014) Sequencing revealed two different compositions of introns in the *cytb* gene in isolates collected from German potato growing areas. An additional intron was found 2 codons upstream of the putative F129L mutation site in the *cytb* gene of genotype I isolates that is absent in genotype II isolates. 63% of in total 203 *A. solani* isolates collected between 2005 and 2011 in Germany were of genotype I. Interestingly, the F129L mutation was exclusively found with a frequency of 97% in genotype II isolates and never in the more abundant genotype I (Leiminger *et al.* 2014). It has not become evident why the F129L substitution could only evolve in genotype II in Germany, whereas it could also evolve in genotype I in the USA, where genotype II is more abundant. Regardless, spore germination assays showed that German F129L *A. solani* mutant isolates had a 4-fold reduced sensitivity to azoxystrobin and a 2.5-fold decreased sensitivity to pyraclostrobin, which is less compared to U.S. isolates. This translated to a roughly 50% loss in Early Blight control efficacy by azoxystrobin in a greenhouse trial. (Leiminger *et al.* 2014)

An emerging problem in Early Blight Control is dual resistance to QoI and SDHI fungicides. A loss of sensitivity of *A. solani* field isolates towards the SDHI boscalid was first reported in 2009 from Idaho in the USA (Wharton *et al.* 2012). A rapid increase in the frequency of boscalid-resistant *A. solani* field isolates from 15% in 2009 to 80% in 2011 was subsequently observed (Miles *et al.* 2014). All tested isolates in 2009 and 2010 were insensitive to azoxystrobin and dual resistance to azoxystrobin and boscalid was recognized as emerging. However, insensitivity to pyraclostrobin was not observable in 2009 and with 22% of tested isolates in 2010 at a comparatively low level (Fairchild *et al.* 2013). Cross-resistance within SDHIs was to some extent observable but was diverse, presumably depending on the molecular spatial structure of the respective SDHI that might influence SDHI binding kinetics to their target enzyme complex succinate dehydrogenase (SDH) (Fairchild *et al.* 2013; Miles *et al.* 2014). SDHIs are, like QoIs, single-site respiration inhibitors but act on complex II instead of complex III of the mitochondrial respiratory chain. The SDH complex (complex II) consists of the four subunits SDHA, SDHB, SDHC and SDHD. Oxidation of succinate to fumarate at SDHA provides additional electrons that are loaded onto ubiquinone at the ubiquinone binding site consisting of residues of SDHB, SDHC and SDHD. Reduced ubiquinone (ubiquinol) subsequently travels to the Qo site of complex III. SDHIs physically interfere with electron transfer from complex II to ubiquinone at the ubiquinone binding site of complex II. Several amino acid substitutions in SDHB, SDHC and SDHD have been found to confer to SDHI resistance in phytopathogens (Avenot and Michailides 2010). In

A. alternata these are H277R or H277Y on SDHB, whereas the amino acid substitutions on SDHB are H278R or H278Y in *A. solani*. Further, the H134R substitution on SDHC and the H133R or the D123E substitution on SDHD were found to confer SDHI resistance in both *Alternaria* spp. (Avenot and Michailides 2010; Mallik *et al.* 2014). SDHB and SDHC mutations were also seen to co-occur in *A. solani* (Landschoot *et al.* 2017) and in *A. alternata* isolates (Avenot *et al.* 2014). SDH mutations are generally indicative of a loss of sensitivity towards SDHIs although the associated phenotypic resistance degree varies within isolates showing a given SDH mutation (Mallik *et al.* 2014; Landschoot *et al.* 2017).

A recent study discovered the presence of SDHI resistance mutations in 41% of *A. alternata* and in 70% of *A. solani* field isolates collected from naturally infected potato fields in Belgium in Central Europe (Landschoot *et al.* 2017). All SDH-mutant isolates were generally less sensitive to boscalid, irrespective of which SDH subunit was mutated. Further, 58% of *A. alternata* and 40% of *A. solani* SDH-mutant isolates simultaneously showed SDHI and QoI resistance mutations.

OBJECTIVES

This study aimed to survey the current status of QoI mutations occurrence in *A. alternata* and *A. solani* populations in major potato growing areas in Bavaria, located in Southern Germany in Central Europe. Further aims were to examine the current frequency distribution of genotype I and genotype II of *A. solani* and to explore to which extent genotype I may have evolved the F129L substitution. An analysis of SNPs causing QoI mutations should generate hints on whether *Alternaria* QoI mutations have evolved independently multiple times. Finally, we aimed to check the presence of SDHI resistance mutations in Bavaria based on the examination of a subset of randomly selected field isolates.

MATERIALS AND METHODS

Collection of isolates

Potato leaves showing *Alternaria* leaf spots were collected during August and September 2016 from commercial and experimental potato fields in the regions Lower Bavaria, Lower Franconia, Swabia, Upper Bavaria and Upper Palatinate which are major potato growing regions in the state of Bavaria located in Southern Germany. Leaves were kept dry at room temperature for a few days until analysis.

Preparation of single spore isolates

From symptomatic leaves, leaf pieces bearing single lesions were excised and surface-sterilized in 5% NaOCl for 1 minute. After washing the leaf cut in sterile, distilled water, it was placed on synthetic low nutrient agar (SNA, 1 g/l KH₂PO₄, 1 g/l KNO₃, 0.5 g/l MgSO₄·7H₂O, 0.5 g/l KCl, 0.2 g/l glucose, 0.2 g/l sucrose, 600 µl 1M NaOH, 22 g/l Agar). To induce sporulation, plates were incubated in a growth cabinet at 20 °C under black light with a 12 h photoperiod for about two weeks. Subsequently, plates were checked for sporulation using a binocular and single spores were transferred to new SNA plates with a fine needle. To obtain single spore colonies, only one conidium of *A. solani*, or a single conidia chain of *A. alternata*, per leaf piece was transferred to fresh SNA plates. Successful isolation of *A. alternata* and *A. solani* cultures was checked microscopically by their characteristic spore morphology and size. The SNA plates were subsequently incubated in a growth chamber at 23 °C under blacklight and 65% relative

Humidity (rH). To obtain pure cultures, the spore isolates were sub-cultured once on SNA plates. Afterwards, small agar plugs of the cultures were transferred to potato-extract dextrose agar (PDA, 4 g/l potato infusion, 20 g/l glucose, 15 g/l Agar) to favor mycelium growth for DNA extraction. The PDA plates were incubated under blacklight for about 30 days at 23 °C and 65% rH until plates were fully colonized with mycelium.

DNA extraction

Genomic DNA was isolated from 80-100 mg mycelium scraped off from fully colonized PDA plates of *A. alternata* and *A. solani* single spore isolates. The fungal mycelium was then transferred into a 2 ml microcentrifuge tube together with 50 µl sterile water and 10-20 glass beads (2 mm diameter, Carl Roth). After the bead-beating step for 3 minutes at 20 Hz (Retsch), DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions (beginning with step 7).

Detection of QoI resistance isolates

The primers AF and AR (Table 1) were used to amplify the QoI mutation prone region of the cytochrome *b* gene in *A. alternata* according to Ma *et al.* (2003). In consequence of the different intron exon composition of the amplified *cytb* gene region in *A. solani* genotype I and genotype II, both the primer pairs As-5f/As-5r for genotype I and As-Gf/As-Gr for genotype II according to Leiminger *et al.* (2014) and Pasche *et al.* (2005), respectively, were used to amplify the appropriate *cytb* gene region for each *A. solani* isolate. PCR reactions were set up in 25 µl volumes containing 1 µl DNA extract, 0.2 µM of each Primer, 0.2 mM of each dNTP (Carl Roth), 1.5 mM MgCl₂, 1x Peqlab Taq-Polymerase Buffer Y and 1 U Taq-Polymerase (Peqlab). PCR conditions are described in Table 1. PCR products were separated by agarose-gelelectrophoresis on a 1.5% agarose gel with 1x Tris-Borate-EDTA-(TBE) Buffer and stained with PeqGreen (Peqlab). The appropriate fragments were excised and purified with the QIAquick Gel Extraction kit (Qiagen). Finally, the purified amplicons were sequenced in both directions using the same primers as for the PCR (GATC Biotech).

Detection of SDHI resistance isolates

PCR reactions were carried out in 25 µl-volumes containing 1 µl DNA extract, 0.2 µM of each Primer, 0.2 mM of each dNTP (Carl Roth), 1x Enhancer Solution P (Peqlab), 1x Peqlab Taq-Polymerase Buffer Y and 1 U Taq-Polymerase (Peqlab). Primers and PCR conditions for amplification of the subunits *SdhB*, *SdhC* and *SdhD* in *A. alternata* as well as *A. solani* are listed in Table 1. Gel extraction and sequencing was done analogous to QoI mutation detection.

Table 1. Primers and PCR conditions

Primer	Target	Reference	PCR parameters
AF	Cyt b, <i>A. alternata</i>	Ma <i>et al.</i> (2003)	95°C for 3 min, 35 cycles (94°C for 40 s, 68°C for 40 s and 72°C for 60 s), 72°C for 10 min
As-Gf	Cyt b, <i>A. solani</i>	Leiminger <i>et al.</i> (2014)	95°C for 10 min, 35 cycles (95°C for 60 s, 54°C for 30 s and 72°C for 30 s), 72°C for 10 min
As-Gr	<i>A. solani</i> genotype I		
As-5F	Cyt b, <i>A. solani</i>	Pasche <i>et al.</i> (2005)	95°C for 10 min, 35 cycles (95°C for 60 s, 54°C for 30 s and 72°C for 30 s), 72°C for 10 min
As-5R	<i>A. solani</i> genotype II		
SdhBa-F	<i>SdhB</i> , <i>A. alternata</i>	Avenot <i>et al.</i> (2008)	95°C for 3 min, 40 cycles (94°C for 40 s, 51°C for 50 s and 72°C for 60 s), 72°C for 10 min
SdhBa-R	<i>A. alternata</i>		
SdhCa-F	<i>SdhC</i> , <i>A. alternata</i>	Avenot <i>et al.</i> (2009)	95°C for 3 min, 40 cycles (94°C for 40 s, 51°C for 50 s and 72°C for 60 s), 72°C for 10 min
SdhCa-R	<i>A. alternata</i>		
SdhDa-F	<i>SdhD</i> , <i>A. alternata</i>	Avenot <i>et al.</i> (2009)	95°C for 3 min, 40 cycles (94°C for 40 s, 51°C for 50 s and 72°C for 60 s), 72°C for 10 min
SdhDa-R	<i>A. alternata</i>		
SdhBs-F	<i>SdhB</i> , <i>A. solani</i>	Mallik <i>et al.</i> (2014)	95°C for 2 min, 30 cycles (95°C for 30 s, 60°C for 30 s and 72°C for 60 s), 72°C for 7 min
SdhBs-R	<i>A. solani</i>		
SdhCs-F	<i>SdhC</i> , <i>A. solani</i>	Mallik <i>et al.</i> (2014)	95°C for 2 min, 30 cycles (95°C for 30 s, 60°C for 30 s and 72°C for 60 s), 72°C for 7 min
SdhCs-R	<i>A. solani</i>		
SdhDs-F	<i>SdhD</i> , <i>A. solani</i>	Mallik <i>et al.</i> (2014)	94°C for 5 min, 40 cycles (94°C for 30 s, 55°C for 30 s and 72°C for 60 s), 72°C for 7 min
SdhCs-R	<i>A. solani</i>		

Supplementary Table 1

RESULTS AND DISCUSSION

Frequency of QoI mutant *A. alternata* and *A. solani* field isolates

In total 55 isolates of *A. alternata* and 47 isolates of *A. solani* were prepared as single spore cultures from diseased potato leaves. The leaves were collected in 2016 from 26 locations distributed in the major potato growing areas in Bavaria in Southern Germany. Up to three independent isolates per location were analyzed. 74.5% of *A. alternata* isolates carried the G143A mutation (Figure 1) which was exclusively caused by a GGT to GCT SNP in their nucleotide sequences of the *cytb* gene. The G143A mutation typically causes a severe loss in QoI sensitivity (Gisi *et al.* 2000; Pasche and Gudmestad 2008). G143A mutant *A. alternata* isolates were found in all examined regions. *A. alternata* G143A mutant frequencies were highest in Upper Palatinate (100%, n = 7), Lower Franconia (100%, n = 2) and Swabia (90%, n = 10), followed by Upper Bavaria (65%, n = 20) and Lower Bavaria (62.5%, n = 16). All multiple *A. alternata* isolates per location were G143A mutated in Upper Palatinate (2 locations) and Lower Franconia (1 location). This was also the case for 3 of 4 locations in Swabia, where the remaining was a mixture of a sensitive and a mutant isolate. This situation was more diverse in the more abundantly sampled regions Upper and Lower Bavaria. Here, locations with all independent isolates being mutated (2 of 7, resp. 3 of 7), locations showing a mixture of sensitive and insensitive isolates (4 of 7, resp. 3 of 7) and locations with all isolates not being mutated (1 of 7 each) were observed.

Percent QoI fungicide mutants

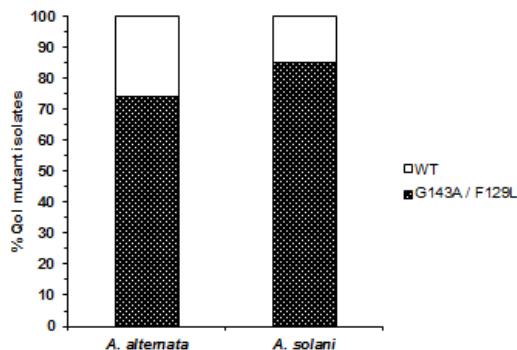


Figure 1. Percent QoI fungicide mutant field isolates

74.5% of $n = 55$ *A. alternata* isolates showed the G143A mutation and 85.1% of $n = 47$ *A. solani* isolates showed the F129L mutation in 2016. WT: wildtype. QoI: Quinone outside inhibitor

The frequency of QoI mutations was comparatively higher in *A. solani* field isolates. 85.1% of analyzed *A. solani* isolates showed the F129L mutation. The F129L mutation causes a loss in sensitivity to QoI fungicides although the degree was seen to vary (Kim *et al.* 2003; Pasche *et al.* 2005). The percentage of *A. solani* F129L mutants was high in all sampled regions. All of the tested isolates carried the F129L mutation in Swabia ($n = 10$) and Upper Palatinate ($n = 8$). 88.2% of isolates were F129L mutated in Lower Bavaria ($n = 17$). The lowest frequency of 63.6% F129L mutant isolates was found in Upper Bavaria ($n = 11$). In Lower Franconia only one sensitive isolate was obtained from one location. This distribution was somewhat reflected by the composition of sensitive and mutant isolates at locations where at least two independent isolates could get prepared. All multiple isolates per locations were F129L mutated in Swabia (3 locations) and Upper Palatinate (3 locations). In Lower Bavaria a mixture of sensitive and mutated isolates was only found in one of 5 locations whereas at the others all isolates were mutated. Finally, three of 5 locations showed a mixture of sensitive and mutated isolates in Upper Bavaria and the remaining showed only F129L mutated isolates.

Together, QoI fungicide resistance mutations are present at high levels in *A. alternata* and *A. solani* field isolates, also in geographically distinct potato growing areas, in Bavaria in Southern Germany. Leiminger *et al.* (2014) found 74% of German *A. solani* isolates, predominantly sampled in Bavaria, to be F129L mutated in 2011. They further observed a patchy distribution of mutant isolates and sensitive isolates in and between regions. Our results show that F129L mutations are now present in all major Bavarian potato growing regions. Our results further indicate that the frequency of the F129L mutation has increased by roundabout 10% in the period between 2011 and 2016. In the Midwest of the USA, it took only five years from the first observed F129L mutant *A. solani* isolates to 96.5% of sampled isolates being mutated (Pasche and Gudmestad 2008). The increase of the F129L mutation frequency in Bavarian *A. solani* isolates appears to progress slower as it has not reached such a high level since 2009. Nevertheless, in the light of 85.1% of *A. solani* and 74.5% of *A. alternata* isolates

showing mutations for reduced sensitivity against QoIs, it appears at least questionable whether there would be an additional benefit in using single compound QoI fungicides for *Alternaria* leaf spot control.

QoI mutations in A. solani genotype I and frequencies of A. solani genotypes I and II

As Leiminger *et al.* (2014) exclusively observed F129L mutations in genotype II of German *A. solani*, isolates, we were interested whether the composition of genotypes in the Bavarian *A. solani* population has changed since 2011 and whether genotype I meanwhile gained the F129L mutation. 25.5% of our analyzed *A. solani* isolates ($n = 47$) belonged to genotype I in 2016 (Figure 2). This resembled the results of Leiminger *et al.* (2014) who found 24.5% ($n = 94$) of German *A. solani* isolates to belong to genotype I in the year 2011, which were mostly collected in Bavaria. They also showed that the frequency of genotype II rapidly increased from 5.1% in 2009 to 75.5% in 2011. Our data indicate that the shift to genotype II has come to a halt and that the distribution of genotypes may have found a balance.

Frequency of *A. solani* genotypes

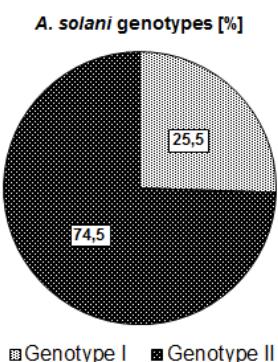


Figure 2. Distribution of *A. solani* genotypes

25.5% of $n = 47$ *A. solani* field isolates were genotype I and 74.5% were genotype II in 2016

Leiminger *et al.* (2014) found F129L mutations with a frequency of 98.6% ($n = 71$) in genotype II isolates in 2011 and never in genotype I. We likewise found 100% ($n = 35$) of *A. solani* genotype II isolates to be F129L mutated in 2016. However, we also found F129L mutations with a frequency of 41.7% ($n = 12$) in genotype I isolates of *A. solani* (Figure 3). Genotype I F129L mutant *A. solani* isolates were identified from Lower Bavaria ($n = 2$), Swabia ($n = 2$) and Upper Palatinate ($n = 1$), but not from Upper Bavaria. This shows that within the five year period between the two sampling time points, genotype I likely has evolved the F129L mutation and that F129L mutant genotype I isolates have undergone positive selection. This further implies that the observed 10% increase of F129L mutant isolates between 2011 and 2016 is very likely due to the evolution of the F129L mutation in *A. solani* genotype I.

F129L in *A. solani* genotypes

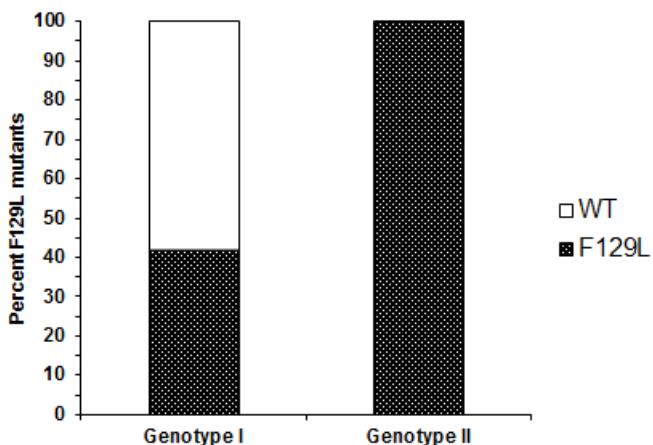


Figure 3. Percent F129L mutant isolates in *A. solani* genotype I and genotype II

41.7% of $n = 12$ genotype I and 100% of $n = 35$ genotype II *A. solani* field isolates showed the F129L amino acid exchange in 2016. WT: wildtype

SNPs causing F129L in *A. solani*

In this study, F129L mutant *A. solani* isolates were collected from all examined Bavarian potato growing regions, which are partly geographically separated. Only seven out of 47 examined *A. solani* field isolates did not carry the F129L mutation. These were genotype I isolates from 5 different sites in Lower Bavaria, Upper Bavaria and Lower Franconia. F129L mutant genotype II isolates were co-isolated from all of these sites except from one site in Lower Franconia where only one genotype I wildtype isolate was available. Together with the previous finding of a patchy distribution of F129L mutants within and between regions five years before by Leiminger et al. (2014), this indicates that the F129L mutation has apparently spatially spread. Therefore we wondered whether we could get hints on the mechanism of the spread by determining the distribution of the SNPs CTC, TTA and TTG (wildtype codon: TTC) that can cause the amino acid exchange from phenylalanine (F) to leucine (L). Diverse SNPs in and between regions would point to multiple independent evolutions whereas the opposite would rather point to a dispersal of F129L mutant *A. solani* isolates.

The TTA SNP was found with 88.6% to be the predominant SNP in *A. solani* genotype II (Figure 4). Further it was the most abundant SNP in each sampled region. In Swabia all F129L mutations in *A. solani* genotype II isolates ($n = 8$) were caused by the TTA SNP. The CTC SNP was found as second SNP to cause the F129L with a frequency of 11.4% of all sampled *A. solani* genotype II isolates. It was present with a frequency of 15.4% ($n=13$) in Lower Bavaria and with 14.3% each in Upper Bavaria ($n = 7$) and Upper Palatinate ($n = 7$). The TTG SNP was never found in the examined genotype II isolates. The TTA SNP having been predominant in all regions might point to a physical spread by e.g. conidia dispersal or by seed potato contamination, although it cannot be excluded that it preferably evolves. However, the CTC SNP found in the

neighboring potato growing regions Lower Bavaria, Upper Bavaria and Upper Palatinate but not in the more geographically separated Swabia indicates at least one independent evolution of the F129L amino acid substitution in parts of Bavaria.

SNPs causing F129L in *A. solani*

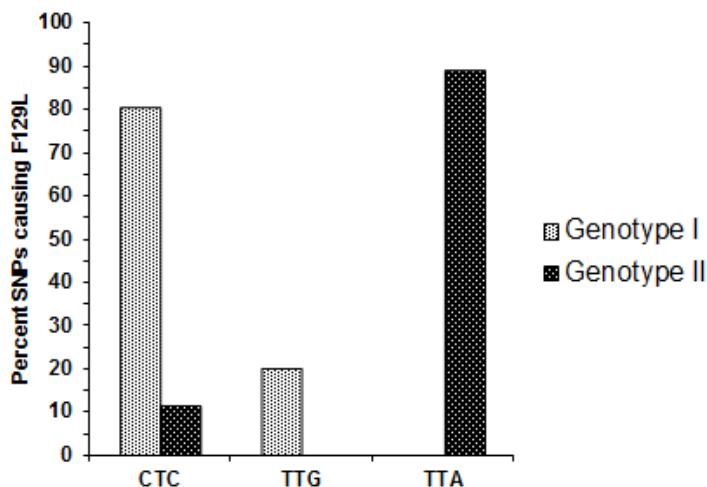


Figure 4 Frequency of SNPs causing F129L in *A. solani* genotype I and genotype II

In genotype I, the F129L amino acid exchange was caused to 80% by the SNP CTC, to 20% by TTG and to 0% by TTA in $n = 5$ field isolates. In genotype II, the distribution of SNPs was 88.6% TTA, 11.4% CTC and 0% TTG in $n = 35$ field isolates. SNP: single nucleotide polymorphism. Wildtype codon = TTC. Exchanged nucleotides are underlined

The CTC SNP is in contrast the most abundant SNP causing F129L in *A. solani* genotype I. In total 80% of F129L mutated genotype I isolates ($n = 5$) showed the CTC SNP, the remaining the TTG SNP, which has not been found in genotype II. The abundantly in genotype II present TTA SNP was not found in genotype I. The CTC SNP was present in F129L mutant *A. solani* isolates in Swabia, Upper Palatinate and Lower Bavaria. The TTG SNP only co-occurred in Lower Bavaria. Like in genotype II, the presence of two different SNPs causing F129L indicates at least one independent evolution of the F129L mutation in *A. solani* genotype I.

The clear dominance of each one distinct SNP in *A. solani* genotype II and genotype I might point to spatial spreading of the F129L mutation as cause for the increase of F129L mutant isolates instead of multiple independent evolutions. However, more research would be required to substantiate that hypothesis. Lower Bavaria could be the hot spot of QoI evolution in Bavaria since it was the sole region where all SNPs causing F129L found in this study were simultaneously present. This first hint would be worth further investigations.

Upcoming SDHI mutations and dual fungicide insensitivity in A. alternata and A. solani

Belgian *A. alternata* and *A. solani* populations showed considerable frequencies in SDH mutations and to some extent also dual mutations against QoI and SDHI fungicides (Landschoot *et al.* 2017). We randomly selected 23 *A. alternata* and 19 *A. solani* of our Bavarian isolates and screened them for the presence of SDHI fungicide mutations in the subunits SDHB, SDHC and SDHD. From the examined isolates, a total of 43.5% of *A. alternata* and 42.1% of *A. solani* isolates showed SDH mutations (Figure 5). All SDH mutated *A. alternata* and *A. solani* isolates exhibited a QoI mutation in parallel. The SDHB mutation was with a frequency of 26.1% (60% within SDH mutant isolates) predominant in the screened *A. alternata* isolates. It was caused in 5 out of 6 cases by the H277Y amino acid exchange and in one case by the H277R substitution. Further, the SDHC mutation was detected with a frequency of 17.4% in *A. alternata*. The SDHD mutation was not observed in *A. alternata*. Also in Bavarian *A. solani* isolates only SDHB and SDHC mutations were detected but the ranking in their frequencies was reverse. The SDHC mutation was with a total frequency of 36.8% (87.5% within SDH mutant isolates) clearly more common than the SDHB mutation (H278Y only) with a frequency of 5.3%.

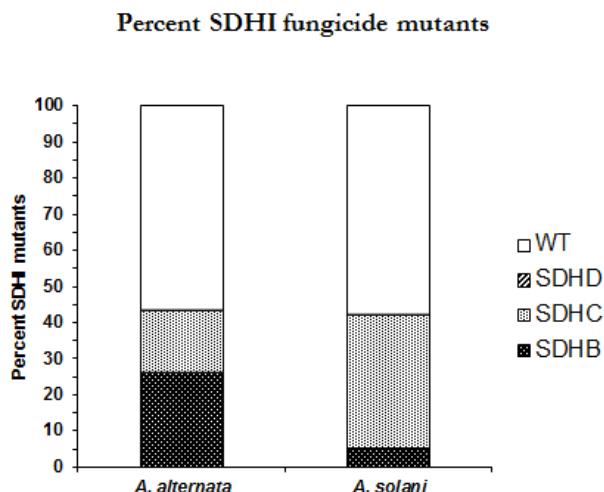


Figure 5. Frequency of succinate dehydrogenase (SDH) mutations in *A. alternata* and *A. solani*. In *A. alternata*, 43.5% of $n = 23$ field isolates showed a SDHI fungicide mutation. The frequencies of mutant isolates were 26.1% SDHB (83.3% H277Y, 16.7% H277R), 17.4% SDHC and 0% SDHD. In *A. solani*, the total frequency of SDH mutated field isolates was 42.1% of $n = 19$. 36.8% of isolates showed a SDHC mutation and 5.3% a SDHB (H278Y) mutation. No SDHD mutation was observed. SDHI: Succinate dehydrogenase inhibitor
SDHB, SDHC, SDHD: Subunits of succinate dehydrogenase enzyme complex. WT: wildtype

The total frequency of SDH mutations in Bavarian *A. alternata* isolates resembled the results from Landschoot *et al.* (2017) where 41% of Belgian isolates were SDH mutated. However, SDH mutations were less prevalent in Bavarian *A. solani* isolates compared to Belgian ones, of which

70% were mutated. This might point to differences in selection pressure exerted on *A. solani* populations. The dominancy and the frequency of the SDHB mutation in *A. alternata* were comparable to the results of Landschoot *et al.* (2017), who found 21.21% SDHB mutated isolates in 2015. In contrast to Belgian isolates in which only the H277Y substitution was found, also a H277R exchange was detected in addition to the predominant H277Y in Bavaria. The balance between SDHB and SDHC mutations was more pronouncedly shifted to SDHC mutations in Bavarian *A. solani* isolates collected in 2016 in comparison to Belgian isolates collected in 2015. Landschoot *et al.* (2017) reported 38.10% SDHC mutations and 26.19% SDHB mutations, of which were 2.38% caused by H278R and the overwhelming part by H278Y. No H278R mutation was detected in Bavarian *A. solani* isolates. This might be due to the reduced number of screened isolated in this study. The same might hold true for the absence of SDHD mutations in our *A. alternata* and *A. solani* isolates. SDHD mutations were found in Belgian *A. alternata* isolates with a comparatively low frequency of 9.09% in 2016 and were absent in Belgian *A. solani* isolates (Landschoot *et al.* 2017). In contrast to Landschoot *et al.* (2017) and Avenot *et al.* (2014) who observed dual mutations of the SDHB and SDHC subunits in Belgian *A. solani* and U.S. *A. alternata* isolates, respectively, we could not observe dual SDH mutations in our set of isolates. However, all SDH mutations being accompanied by QoI mutations in Bavarian *A. solani* isolates resembled more the situation in the USA where 99% of screened SDH mutated isolates showed the F129L mutation in parallel (Gudmestad *et al.* 2013) than the situation in Belgium where only 28.76% of isolates showed dual mutations against SDHIs and QoIs and where isolates only being SDH mutated were with 39.76% even predominant (Landschoot *et al.* 2017). This contrast between Bavarian and Belgian isolates was also observed for *A. alternata*, where in Belgium only 44.23% of the in 2015 collected isolates showed dual mutations against QoIs and SDHIs. These differences could possibly be explained by different applied fungicide strategies and active ingredient combinations.

In the overall view, it can be stated that QoI fungicide resistance mutations are fully established in the major potato growing areas in Bavaria. The results of this study further show that dual resistance against QoI and SDHI fungicides are an upcoming problem in chemical *Alternaria* disease control in potatoes in Bavaria. This is reflected by reports that reach us from agricultural practice.

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