

Evidence of strobilurine resistant isolates of *A. solani* and *A. alternata* in Germany

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SUMMARY

Early blight caused by *Alternaria solani* and *Alternaria alternata* is a highly destructive disease of potatoes. Control of early blight mainly relies on the use of preventive fungicide treatments. Because of their high efficacy, QoI fungicides are commonly used to control early blight. However, loss of sensitivity to QoIs has been reported for *A. solani* in potato (Pasche *et al.* 2001) and for *A. alternata* in different hosts (Vega & Dewdney, 2014).

Five hundred and five *A. solani* field isolates collected from 150 locations in Germany between 2005 and 2014 were screened for the presence of the F129L mutation in the cytochrome *b* gene; of these, 147 contained the F129L mutation. Sequence analysis revealed the occurrence of two structurally different *cytb* genes. F129L isolates have been found since 2009 onward. Sensitivity of *A. solani* isolates to azoxystrobin was determined in conidial germination assays. All *A. solani* isolates possessing the F129L mutation had reduced sensitivity to azoxystrobin. The results indicated an increase of the EC₅₀-value over the last three years.

In identical form two hundred and eight *A. alternata* field isolates were collected from 101 locations in Germany between 2005 and 2014. These isolates were screened for the presence of the G143A mutation in the cytochrome *b* gene; of these, 106 contained the G143A mutation.

Sensitivity of *A. alternata* isolates to azoxystrobin was determined in conidial germination assays. As well all *A. alternata* isolates possessing the G143A mutation had highly reduced sensitivity to azoxystrobin.

KEYWORDS

Alternaria solani, *Alternaria alternata*, cytochrome *b*, fungicide resistance, QoI

INTRODUCTION

Potato early blight occurs worldwide and is prevalent wherever potatoes are grown. Early blight of potato (EB), caused by the fungi *Alternaria solani* and *Alternaria alternata*, can be found in all

German potato growing areas. The disease is a risk to crop productivity in the field and results in significant yield losses. Integrated pest management is mainly based on multiple fungicide applications. Prior to the registration of azoxystrobin (QoI) for potato in 2007 in Germany, EB control was mainly achieved by multiple and frequent application of mancozeb-containing fungicides. However, these fungicides were only moderately effective in the control of EB. Ortiva[®] (azoxystrobin) and Signum[®] (pyraclostrobin+boscalid) were registered in Germany as EB-specific fungicides in potatoes in 2007 and 2008, respectively. In contrast to mancozeb-containing fungicides, QoI fungicides turned out to be highly effective against EB. Strobilurins (QoIs) inhibit mitochondrial respiration in fungi by binding to the Qo site of the cytochrome *b* (*cytb*) complex, blocking electron transfer and inhibiting ATP synthesis. As QoI's have a specific single-site mode of action they possess a high risk to the evolution of fungicide resistance due to point mutations.

The occurrence of fungicide resistance is a serious problem affecting disease management in agricultural ecosystems. The knowledge about the occurrence of reduced-sensitive isolates is necessary for an integrated pest management.

The present study was carried out in order to determine whether a reduction in sensitivity to azoxystrobin has already occurred within German *A. solani* and *A. alternata* populations. The objectives of this research were to determine the prevalence of the F129L substitution among *A. solani* and the G143A substitution among *A. alternata* isolates collected from conventional potato fields throughout Germany between 2005 and 2014.

MATERIALS AND METHODS

Isolate sampling

Between 2005 and 2014 505 *A. solani* were collected from 150 locations and 208 *A. alternata* field isolates from 101 locations in Germany. All isolates originated from commercial potato crops naturally infected with EB. Infected leaflets were sampled gathered at random during EB disease epidemics between July and September of each year. Material was surface-sterilized in 5% NaOCl for 1 min and then washed in sterile, distilled water. One single conidium was transferred to synthetic low nutrient (SN) medium (1 g KH₂PO₄; 1 g KNO₃; 0.5 g MgSO₄·7H₂O; 0.5 g KCl; 0.2 g glucose; 0.2 g saccharose; 0.6 ml 1 n NaOH; 20 g agar; dissolved in 1,000 ml double distilled water). Only one isolate per diseased plant was collected. *Alternaria solani* or *Alternaria alternata* cultures were identified on the basis of morphological characteristics and spore size (Simmons, 2007).

Evaluation of the F129L substitution in A. solani and the G143A substitution in A. alternata

Genomic DNA of *A. solani* and *A. alternata* isolates was extracted from fungal mycelia cultivated on V8-medium under near ultraviolet light for 14 days at 21°C.

For genomic DNA extraction, *A. solani* and *A. alternata* isolates were cultivated on V8-medium under 12 h near ultraviolet light for 14 days at 21°C.

Mycelium and spores were carefully scraped off with a spatula and ground in liquid nitrogen. Genomic DNA extraction, PCR, probe hybridization and sequencing of a cytochrome *b* fragment for the detection of the F129L mutation was carried according to Leiminger *et al.* (2013).

In vitro QoI fungicide sensitivity assay

Fungicide sensitivity was determined using an *in vitro* plate assay, based on germination rate of conidia on fungicide-amended agar relative to non-amended media. Spores of *A. solani* and

A. alternata were produced by growing isolates on SN media at 20°C with an alternating 12 h photoperiod. Conidia were dislodged using a sterile glass rod and distilled H₂O. The conidial suspension of each isolate was spread onto the surface of fungicide-amended agar plates at different concentrations, as well as on fungicide free plates which served as control plates. After incubation of the plates, the germination of 100 conidia per plate was assessed.

Fungicide sensitivity was determined by comparing spore germination on water agar amended with azoxystrobin at different concentrations. Fungicide sensitivity assays were performed in the presence of 100 mg/l salicylhydroxamic acid (SHAM). SHAM was added to media to prevent the fungus from overcoming the toxicity of a QoI fungicide through an alternative oxidative pathway. Petri dishes with SHAM but without fungicides were used as controls. Fungicide sensitivity was measured as the concentration at which spore germination of *A. solani* was inhibited by 50% relative to the untreated control (EC₅₀ value) and was determined for each isolate.

RESULTS

Detection of German A. solani field isolates carrying the F129L substitution

None of the *A. solani* isolates collected between 2005 and 2008 contained the F129L mutation. Two isolates, which contained the F129L mutation, were first observed in 2009. Both derived from the same field in Bavaria. In 2010, two further F129L isolates were sampled from two different locations, whilst in 2011 six different locations (all in Bavaria) showed F129L isolates. In 2011, the number of isolates carrying the F129L mutation increased strongly. Thirty-nine out of 66 isolates (56%) contained the F129L mutation (see Table 1). In 2012 fifteen out of 88, 2013 69 of 183 and in 2014 20 out of 62 isolates contained the mutation. Within F129L isolates, the TTC to TTA nucleotide exchange was predominantly found. A CTC mutation was detected in only three isolates. The frequency of wild type isolates was 100% between 2005 and 2008, 95% in 2009, 80% in 2010 and 28% in 2011. Since 2013 the frequency of F129L mutants has been found as relatively stable between 32 and 37% (Table 1).

Table 1. Details of *Alternaria solani* isolates collected from commercial potato crops in Germany, 2005 -2014

year	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Nr. of isolates	5	24	20	5	39	13	66	88	183	62
Nr of locations	3	22	13	3	22	9	12	14	25	27
Locations with F129L	0	0	0	0	1	2	7	6	20	18
Nr of F129 L mutants	0	0	0	0	2	2	39	15	69	20
Frequency F129L %					5,1	15,3	56,5	17,0	37,7	32,2

Detection of German A. alternata field isolates carrying the G143A mutation

From 2005 to 2014, 208 monoconidial isolates of *A. alternata* from 101 different locations were examined for the presence of the G143A mutation. For the detection of the G143A mutation 208 single-spore isolates from the years 2005 to 2014 and 101 different locations were

examined. In 2006 the first G143A mutant was found in *A. alternata* for Germany. In this year nine out of 43 isolates (21%) contained the G143A mutation. Since 2009 the frequency of G143A mutants has increased over 41%.

The molecular basis for the analysis of QoI resistance was carried out by using polymerase chain reaction of the cytochrome b gene. Within G143A isolates of *A. alternata*, only GGT the GCT nucleotide exchange has been detected so far.

Table 2. Details of *Alternaria alternata* isolates collected from commercial potato crops in Germany, 2005 -2014

	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	in total
isolates evaluated	7	43	/	/	14	12	30	34	17	51	208
wildtyp isolates	7	34	/	/	5	0	7	20	7	22	102
G143A mutants	0	9	/	/	9	12	23	14	10	29	106
investigated locations	1	29	/	/	10	1	11	10	4	35	101
locations with G143A	0	9	/	/	5	1	8	5	0	18	46

In vitro assay of *A. solani* isolate sensitivity to QoI fungicides

Fungicide sensitivity was determined by assessing spore germination for a range of isolates on fungicide-amended and non-amended water agar in the presence of SHAM. EC₅₀ values of *A. solani* baseline isolates collected in 2006 ranged from 0.058 to 0.1 µg ml⁻¹ for azoxystrobin (Fig. 1). EC₅₀ values for *A. solani* wildtype isolates, collected after registration of azoxystrobin between 2007 and 2014, were not significantly different from the mean EC₅₀-values of the baseline isolates.

F129L isolates collected 2009-2014 had significantly higher EC₅₀ values compared to the baseline isolates. All isolates possessing the F129L mutation were less sensitive to azoxystrobin. Comparing mean EC₅₀ values, isolates lacking F129L were approximately 4-fold more sensitive compared to F129L isolates collected from 2009 to 2012 (Fig. 1). Since 2013 the mean EC₅₀ values of the F129L isolates have increased dramatically. The mean EC₅₀ value of the F129L mutants collected in 2013 was 1.5 µg ml⁻¹ for azoxystrobin, in 2014 10.2 µg ml⁻¹.

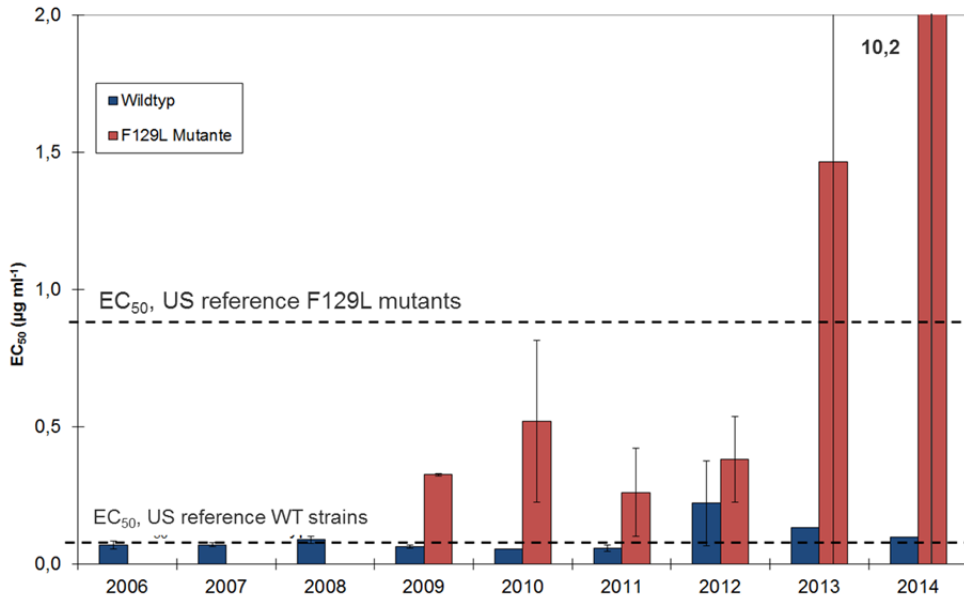


Figure 1. *In vitro* azoxystrobin sensitivity assay of *A. solani* wild-type and F129L isolates collected between 2006 and 2014. Columns represent mean EC_{50} values, i.e. the effective fungicide concentration that inhibited spore germination by 50%. Bars represent standard deviations

In vitro evaluation of isolate sensitivity of *A. alternata* to QoI fungicides

The germination rate of almost all *A. alternata* isolates evaluated in fungicide untreated control plates was over 90%. Compared to this the germination rate in the wild-type isolates was reduced to 8 – 65% by adding $0.1 \mu\text{g ml}^{-1}$ azoxystrobin. At a concentration of $1.0 \mu\text{g ml}^{-1}$ azoxystrobin the germination rate of wild-type isolates was less than 2% (Fig. 2).

By comparing wild-type and G143A isolates, G143A mutants showed a high germination rate at a concentration of $1.0 \mu\text{g ml}^{-1}$ azoxystrobin and even at $10.0 \mu\text{g ml}^{-1}$ azoxystrobin.

The mean EC_{50} values of the *A. alternata* mutant were at least 100 times higher than the mean EC_{50} values of the *A. alternata* wild-type isolates. Figure 3 indicates the fungicide sensitivity of *A. alternata* G143A mutants.

Germination %

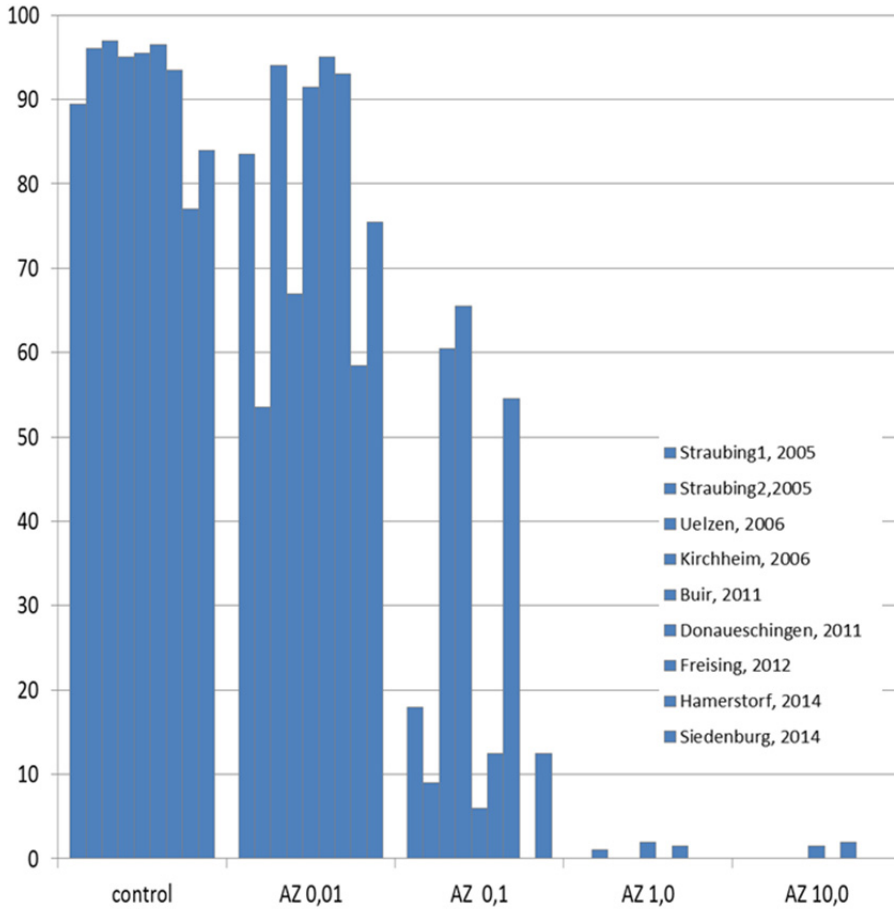


Figure 2. Germination rate of *A. alternata* wild-type isolates from different locations by adding different fungicide-concentrations (AZ: azoxystrobin)

Germination %

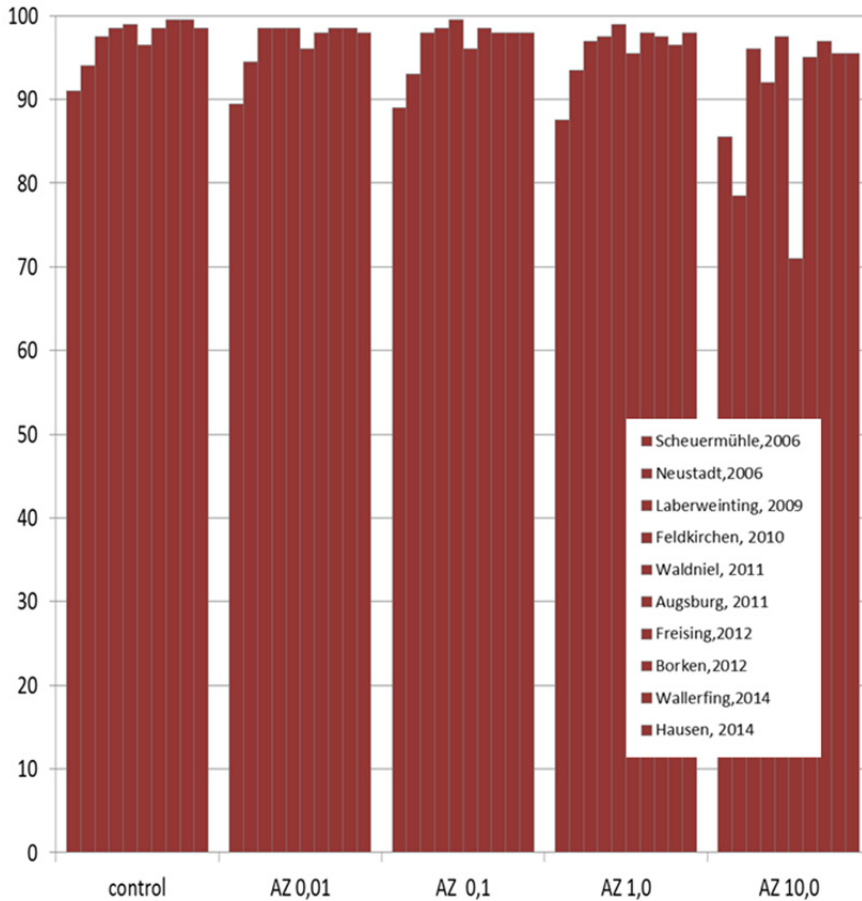


Figure 3. Germination rate of *A. alternata* G143 isolates from different locations by adding different fungicide-concentrations (AZ: azoxystrobin)

CONCLUSION

Resistance and reduced sensitivity to fungicides among fungal plant pathogens are significant problems concerning chemical pest management. QoI fungicides, which are widely used to protect crops, have been characterized as “high-risk” for the development of resistance. In Germany, QoIs were registered for the control of EB in potatoes in 2007 for the first time. First G143A isolates of *A. alternata* were found in 2006, before azoxystrobin was registered for application in potato. For the fungus *A. solani* first F129L isolates were found in 2009, two years after the first registration of azoxystrobin for potatoes in Germany. In the present study, isolates containing the F129L substitution displayed a shift in sensitivity to azoxystrobin in *in vitro* spore

germination assays. The restricted use of QoIs, in combination with management programs will be essential for the continued use of QoI fungicides for the control of EB.

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