PAGV – SPECIAL REPORT NO 18 – 2017, 91-98

Prevalence of QoI and SDHI fungicide resistance in *Alternaria solani* – the situation in Germany

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SUMMARY

Early blight (EB) caused by *Alternaria solani* is a highly destructive disease of potatoes. It is controlled by multiple preventive fungicide treatments during the growing season. QoI and SDHI fungicides are currently most effective but have a single site mode of action. The sensitivity of *A. solani* can be reduced by mutations in the genes of the target site of both fungicide groups. The F129L mutation in the Cyt b gene and mutations in the B, C and D subunits of the SDH are known to cause loss in sensitivity to QoIs respectively SDHIs (Pasche et al., 2005, Mallik et al., 2014).

1151 *A. solani* single spore isolates from the years 2005 to 2016 were screened for the presence of the F129L mutation and mutations in the SDH subunits B, C and D. F129L mutant isolates were first found 2009 in Southern Germany but until 2013 they could be detected in all potato growing areas in Germany. 2015 the percentage of mutant isolates within the population rose to almost 90% and they occurred in every location samples were taken from. *A. solani* SDHI mutants were first detected in 2013. 2014 they occurred in all potato growing areas in Germany but their number was low. This changed 2015 when approximately two-thirds of the surveyed isolates were SDH mutants and they were found in almost 90% of the locations.

In vitro and in vivo sensitivity tests showed a significant loss in sensitivity towards Azoxystrobin (QoI) and Boscalid (SDHI) in F129L respectively SDHI mutant isolates.

Considering the spreading and increase in the number of F129L and SDHI mutant *A. solani* isolates it is obvious that fungicide treatment strategies have to be adapted to maintain the effectiveness of QoI and SDHI fungicides against EB.

KEYWORDS

Alternaria solani, fungicide resistance, cytochrome b, F129L, QoI, SDHI

INTRODUCTION

Alternaria solani is the causative agent of early blight of potato (*Solanum tuberosum*). This very common disease, which can be found in most potato growing countries, can cause considerable

defoliation (Woudenberg et al., 2014). It typically reduces yields by ~20%, but yield reductions of up to 80% have been reported (Horsfield et al., 2010).

In Germany early blight occurs in all potato growing areas. It is controlled by multiple applications of protective fungicides during the growing season.

One of the most effective and therefore frequently applied protective fungicide agents is Azoxystrobin. It belongs to the class of the quinone outside inhibitors (QoIs), which bind to the quinone outside pocket of the cytochrome bc1 complex in the mitochondria and inhibits thus the electron transport in mitochondrial respiration. This single site mode of action implies a high risk of resistance development (FRAC Code List ©*2017) due to point mutations in the cytochrome b gene. In the USA only two years after the registration of Azoxystrobin in potato, Pasche et al. (2004) were the first to report of reduced sensitivity in *A. solani* isolates and demonstrated that this reduction is caused by the F129L mutation in the cyt b gene (Pasche et al., 2005). Leiminger et al. (2014) detected within the *A. solani* populations in Europe two types of cyt b genes which differ in their intro-exon structure.

Another effective protective fungicide is boscalid. It belongs to the class of succinate dehydrogenase inhibitors (SDHIs) which interfere with the mitochondrial respiration, too. They inhibit electron transport at complex II (succinate-dehydrogenase) by binding to the ubiquinone binding site formed by SDH subunits B, C and D. This single site mode of action also has a high risk of resistance development (FRAC Code List ©*2017). Point mutations leading to amino acid changes and reduced sensitivity of *A. solani* isolates can be detected in the gene sequences of all three subunits. Currently described mutations in *A. solani* are in subunit B: H278Y and H278R, C: H134R and in D: D123E, H133R (Mallik et al., 2014). In the USA boscalid was registered for the use in potato in 2005 (Mallik et al., 2014) and 2009 the first insensitive isolates occurred (Wharton et al., 2012).

In Germany Azoxystrobin and Boscalid were registered for the use in potato 2006 and 2008 respectively. The aim of this work was to survey the presence of the F129L mutation concerning the sensitivity towards QoIs and the presence of SDH subunit B, C and D mutants concerning the sensitivity towards SDHIs in German *A. solani* populations. Furthermore, the impact of the different mutations on the sensitivity of *A. solani* isolates towards Azoxystrobin respectively Boscalid was tested both in vitro (data not shown) and in vivo.

MATERIAL AND METHODS

Isolates

1151 *A. solani* single spore isolates from the years 2005 to 2016 were screened for the presence of the F129L mutation and mutations in the SDH subunits B, C and D. They were obtained from leaf samples from 214 locations from all potato growing areas in Germany. The samples were naturally infected and derived mostly from commercial potato crops but also from field trials. Up to ten isolates per sample were taken according to the method published by Leiminger et al. (2014).

DNA extraction

Genomic DNA of the *A. solani* isolates was extracted according to Leiminger et al. (2014).

Detection of the F129L mutation

Within *A. solani* populations in Europe two types of cyt b genes exist which differ in their intro-exon structure (Leiminger et al., 2014).

Therefore a standard PCR with two primer sets was used for the detection of the F129L mutation. Primer pairs As-Gf/r (Leiminger et al., 2014) and As-5f/r (Pasche et al., 2005) were used to amplify DNA segments containing the possible mutation site for genotype I respectively genotype II cyt b genes. The 207/214 bp products were separated by gel electrophoresis, excised, purified and sequenced (Leiminger et al., 2014). PCR for genotype I (As-Gf/r) was carried out according to Leiminger et al., 2014. PCR for genotype II (As-5f/r) was performed in a total volume of 20 µL containing10x PCR buffer, 3,5 mM $MgCl_2$, 200 µM each dNTP (Fermentas), 0,5 µM As-5f primer, 1 µM As-5r primer, 1 U Taq DNA polymerase (SupraTherm, 5 U μL^{-1}, GeneCraft) and 50 ng genomic DNA as template. Cycling conditions were: Initial denaturation step at 95°C for 10 min, followed by 36 reaction cycles consisting of denaturation at 95°C for 1 min, primer annealing at 58°C for 30 s and DNA extension at 72°C for 30 s. After a final extension step of 72°C for 3 min, samples were cooled at 4°C.

Detection of SDH subunit B, C and D mutations

To detect possible mutations in the B, C and D subunits of the SDH complex, a standard PCR with three different primer pairs (SDHB-F/R, SDHC-F1/R2 and SDHD-F1/R2 (Mallik et al., 2014)) was used to amplify the almost complete respective genes. Each PCR was performed in a total volume of 25 µl containing 10x PCR buffer, 1,5 mM $MgCl_2$, 200 µM each dNTP (Fermentas), 0,6 µM forward/reverse primer, 1 U Taq DNA polymerase (SupraTherm, 5 U μL^{-1}, GeneCraft) and 50 ng genomic DNA as template. Cycling conditions for the SDHB gene were: initial step of 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min. After a final extension step at 72°C for 7 min, samples were cooled at 4°C. For the SDHC and D gene the duration of the final extension step was reduced to 45 s. The 1.082, 570 and 607 bp products were separated by gel electrophoresis, excised, purified and sequenced according to Leiminger et al. (2014).

In vivo fungicide sensitivity assay

The effect of the presence of the F129L and SDHI mutations in *A. solani* isolates on the fungicide efficacy of Azoxystrobin and Boscalid was determined in greenhouse trials with inoculated potato plants as described by Leiminger et al. (2014). The sensitivity tests for Azoxystrobin included a subset of six wild-type and five F129L isolates, the tests for Boscalid three wild-type, one B-H278R, two new, not yet described C and one C-H134R SDH mutant isolates.

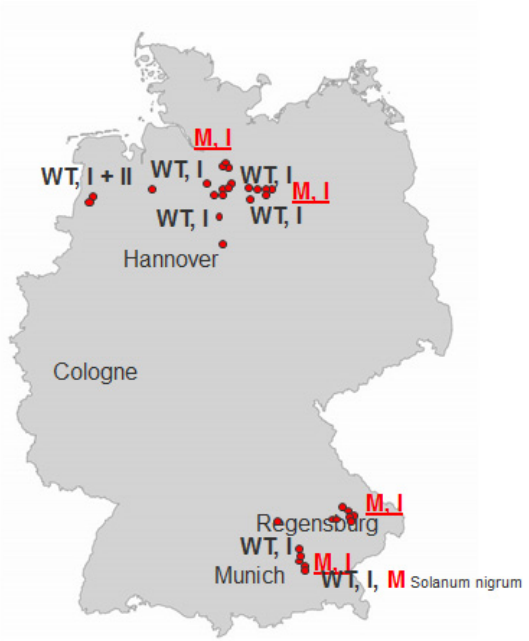

Figure 1. *Occurrence of A. solani F129L mutant isolates in Germany, 2015. Map of locations screened for F129L mutants. Unlabeled dots indicate the presence of genotype II mutant isolates in a location, circles the absence. M, I = genotype I mutants detected. WT, I/II = wild-type genotype I/II detected.*

RESULTS

Presence of F129L mutants in the Alternaria population in Germany

All fifty-five isolates screened for the F129L mutation from 2005 to 2008 were wild-type and with one exception genotype I. They originated from forty locations, most of them in Southern Germany but to a smaller part in Northern and Western Germany, too. In 2009 39 isolates from 21 plots in Southern and Northern Germany were screened and the first two F129L mutant isolates found in one location in Southern Germany, both of them genotype II. In the following year the F129L mutation could be detected in two more locations in Southern Germany, 2011 in eight. 2012 F129L mutant isolates occurred additionally in three locations in Northern Germany and for the second time since 2006 genotype II wild-types in one location each in Southern and Northern Germany. 2013 the first mutant isolates were found in Eastern and Western Germany, so the F129L mutation was present in all potato growing areas in Germany now. Until 2012 F129L mutant isolates were always genotype II but 2013 the first genotype I mutant isolates could be detected. They derived from three different plots in Northern Germany. In total 266 isolates from 24 locations were screened and 72 mutants found in 20.

Of the 67 *A. solani* isolates surveyed 2014 20 had the F129L mutation and 18 out of 30 plots situated in all potato growing areas in Germany were affected. In 2015 a strong increase in the occurrence of the F129L mutation could be observed (Figure 1). 170 isolates out of 196 carried the mutation. They were detected in all 34 plots located in Northern and Southern Germany.

Genotype I mutants occurred in two places each in Northern Germany and Southern Germany. Wild-types could only be found in 7 places at all, one of them for the third time in 10 years a genotype II wild-type isolate. It was also possible to isolate F129L mutants from leaves of *Solanum nigrum* plants which grew nearby a potato crop. The situation in 2016 was similar to 2015: F129L mutant isolates were detected in all 26 plots surveyed and they predominated. Genotype I mutants occurred in three different locations in Northern Germany.

Sensitivity of A. solani isolates towards Azoxystrobin

In vitro plate tests with technical grade Azoxystrobin showed significantly reduced conidia germination rates in F129L mutant isolates (Leiminger et al., 2014). Therefore the fungicide efficacy was tested in vivo in greenhouse trials with inoculated potato plants, too (Figure 2). F129L mutant isolates showed a significantly reduced sensitivity compared to wild-types. At a concentration of 100 µg technical grade Azoxystrobin/ml fungicide spray solution (= field concentration) the efficacy was reduced to 74 to 43% in mutants, whereas wild-type isolates had efficacies around 95%.

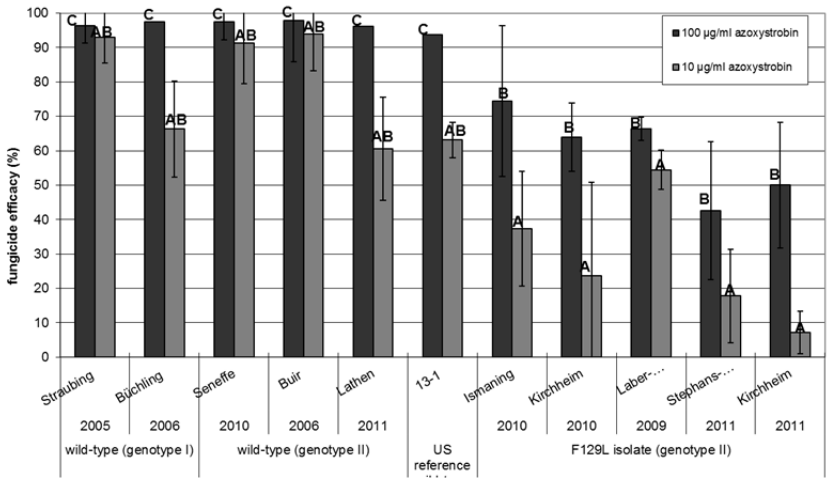

Figure 2. *Fungicide efficacy of Azoxystrobin against German wild-type (n = 6) and F129L (n = 5) Alternaria solani isolates. In vivo greenhouse tests (mean of two replications) with concentrations of 10/100µg technical grade Azoxystrobin/ml fungicide spray solution. Disease severity was rated 1 week after artificial inoculation. Columns with the same letter are not significantly different (Tukey's b test, P = 0_05). Vertical bars indicate standard deviation.*

Presence of SDH mutants in the Alternaria population in Germany

The isolates screened for the F129L mutation were used to survey the presence of SDH mutants in the *Alternaria* population in Germany, too. Within the 297 isolates of the years 2005 to 2012 no SDH mutants could be detected. The first mutants occurred 2013 (Fig.: 3A) in two locations in Western Germany (subunit B: H278Y and subunit C: H134R)) and one location in Northern Germany (subunit C: H134R). 2014 the C-H134R mutation was detected in two locations in Eastern Germany and a new mutation in subunit C, not described in the literature yet, in one plot in Southern Germany (Fig.: 3B).

The situation in 2015 (Fig.: 3C) showed similar to the development concerning the presence of the F129L mutation a dramatic increase in the occurrence of SDH mutant *A. solani* isolates. 134 of the 196 screened isolates showed a mutation in one of the three SDH subunits. 30 out of 34 locations were affected. The predominant mutation was C-H134R but B-H278Y and B-H278R could also be detected as well as the new C and D-D123E mutants. All isolates with a SDH mutation possessed the F129L mutation additionally.
In all of the 26 locations the samples derived from in 2016, SDH mutants were detected. Wild-type isolates only in 4. C-H134R was the predominant mutation but B-H278Y occurred, to a lesser part, too. B-H278R, the new C and D-D123E mutants could not be found in 2016.

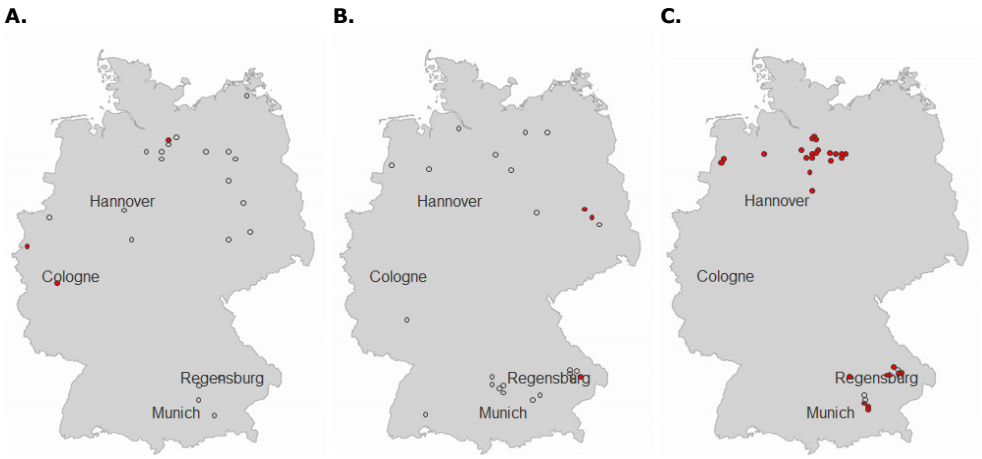

Figure 3. *Occurrence of A. solani SDH mutant isolates in Germany, 2013 (**A**), 2014 (**B**) and 2015 (**C**). Map of locations screened for SDH mutants. Dots indicate the presence of mutant isolates in a location, circles the absence.*

In vivo sensitivity of A. solani isolates towards Boscalid

The three wild-type, one B-H278R, two new C and one C-H134R SDH mutant isolates used for the in vivo sensitivity tests derived from the years 2014 and 2015. At a concentration of 100µg technical grade Boscalid/ml fungicide solution, which matches the field application dose, the SDH wild-type isolates showed a fungicide efficacy of nearly 100%. The mutants were within a range of 98 to 66%. Only the C-H134R isolate differed significantly (Tukey-b, α=5%) from the wild-types.

CONCLUSION

QoI and SDHI fungicides are important tools for the control of early blight caused by *A. solani* in potato. Their single site mode of action holds a high risk of resistance development (FRAC Code List ©*2017) caused by point mutations in the genes encoding the binding sites of the fungicides/ubiquinone. To prevent a fast development, selection and spreading of less sensitive mutants it is important to adapt the fungicide treatment strategy. Therefore it is necessary to get information about the current situation concerning the occurrence of *A. solani* F129L and SDH mutant isolates.

In Germany the first F129L mutant isolates were detected in 2009, three years after the registration of Azoxystrobin for the use in potato, in Southern Germany. Until 2014 the F129L mutation could be found in all potato growing in Germany. Almost all mutant isolates were genotype II, whereas most wild-type isolates were genotype I. 2013 the first genotype I mutants occurred and their number increased until 2016. In 2015 the percentage of wild-type isolates decreased dramatically, almost 90% of the screened isolates were F129L mutants and they were detected in all locations surveyed. Only in 17,6% of the locations wild-type isolates were found. The situation in 2016 was similar.

Sensitivity tests with German *A. solani* F129L mutant isolates in vitro (Leiminger et al., 2014) as well as in vivo showed a significantly reduced sensitivity towards Azoxystrobin compared to wild-types with fungicide efficacies between 74 and 42%.

The first *A. solani* isolates with mutations in the SDH complex were detected in 2013, five years after registration of Boscalid for the use in potato, in North and West Germany. In the following year SDH mutants turned up in East and South Germany, the South German isolates being a new not yet described C mutant. In both years the percentage of mutants within the total amount of screened isolates and the number of affected locations was low. 2015 the situation changed completely: Approximately two-thirds of the surveyed isolates were SDH mutants and they were found in almost 90% of the locations. C-H134R was the predominant mutation but the new C was also detected as well as both known subunit B mutations and D-D123E. 2016 mutants were detected in all surveyed locations, the main mutation being C-H134R and to a lesser part B-H278Y.

SDH C-H134R, C new and B-H278Y mutants tested in vivo for their sensitivity to Boscalid had a reduced sensitivity within a range of 66,5 to 98,3% fungicide efficacy.

A. solani isolates with reduced sensitivity to Azoxystrobin and Boscalid are therefore present in all potato growing areas in Germany and their part in the *A. solani* population is high. Spraying strategies have to be adapted to this situation.

REFERENCES

FRAC Code List ©*2017. http://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2017-final.pdf?sfvrsn=fab94a9a_2

Horsfield, A., Wicks, T., Davies, K., Wilson, D., Paton, S. (2010). Effect of fungicide use strategies on the control of early blight (*Alternaria solani*) and potato yield. Australas. Plant Pathol. 39: 368-375.

Leiminger, J.H., Adolf, B., Hausladen, H. (2014). Occurrence of the F129L mutation in *Alternaria solani* populations in Germany in response to QoI application, and its effect on sensitivity. Plant Pathology 63: 640–650.

Mallik, I., Arabiat, S., Pasche, J.S., Bolton, M.D., Patel, J.S., Gudmestad, N.C. (2014). Molecular characterization and detection of mutations associated with resistance to succinate dehydrogenase-inhibiting fungicides in *Alternaria solani*. Phytopathology 104: 40-49.

Pasche, J.S., Wharam, C.M., Gudmestad, N.C. (2004). Shift in sensitivity of *Alternaria solani* in response to QoI fungicides. Plant. Dis. 88: 181-187.

Pasche, J.S., Piche, L.M., Gudmestad, N.C. (2005). Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. Plant Dis. 89: 269-278.

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 Dis. 96:454.

Woudenberg, J.H.C., Truter, M., Groenewald, J.Z., Crous, P.W. (2014). Large-spored *Alternaria* pathogens in section *Porri* disentangled. Studies in Mucol. 79: 1-47.