Efficacy loss of strobilurins used in protection against apple scab in Czech orchards

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Abstract

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The apple scab caused by *Venturia inaequalis* is important apple disease in temperate zone. Economical losses caused by the pathogen are effectively reduced by intensive chemical protection and breeding. Occurrences of pathogen resistance to strobilurin fungicides were noted in some Czech orchards in the past. In 2007–2011, collection of 136 isolates *V. inaequalis* was tested with the aim to detect presence of single-point mutations of cytochrom *b* (*cytb*) gene causing efficacy loss of strobilurins. Consequently occurrence of beta-tubulin (*beta-tub*) gene mutation causing resistance to benomyl was studied with the aim to obtain comparative data about frequency of mutation in non-selective environment. Therefore simple and highly repeatable PCR based mutation detecting methods were optimised. Analysis brought knowledge about high similarity of *cytb* and *beta-tub* genes mutations occurrence. Whilst the frequency of the G143A substitution in *cytb* gene was 65% the E198A substitution of *beta-tub* gene occurred in 54% within pathogen population. This high frequency of resistant population necessitates revision of the way of strobilurins use in apple scab management.

Keywords: *Venturia inaequalis*; resistance to fungicides; cytochrom *b* gene; PCR based detection of mutations

Apple scab is the major disease in apple tree orchards worldwide caused by ascomycete *Venturia inaequalis* (Cooke) Winter. Since the production of apples currently depends on chemical protection, breeding of new cultivars resistant to *V. inaequalis* is developing for long time as possible solution reducing the usage of chemicals in apple protection (Williams, Kuc 1969). Resistance of cultivars based on *Rvi6* (*Vf*) gene was overcome by pathogen races 6 and 7 in orchards across Europe (Parisi et al. 1993; Roberts, Crute 1994; Bénaouf, Parisi 2000; Bus et al. 2005). The first break of *Rvi6* (*Vf*) determined resistance in the Czech Republic was recorded in four separate locations in 2006. Also chemical management of scab is exposed to enormous pathogen adaptation ability. Occurrence of pathogen resistance to fungicide is an important issue in modern agriculture. Strobilurins and benzimidazoles are examples of single-site inhibitors for apple scab protection, which were released to the market in the late 1970's and in the mid 1990's. In 2011 the total consumption of strobilurins was

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430 kg what presents 0.3% from of 148.8 t of total consumption of fungicides used in fruit plants protection in the Czech Republic. In five years period from 2007–2011, the total strobilurins consumption was relatively stabilised. Strobilurins belong to important class of fungicides in plant protection against many phytopathogenic fungi. They have been successfully used in Europe to control the apple scab since 1998. They are known as Quinone outside Inhibitors (QoI) influencing respiration pathway controlled by *cytb* gene (Bartlett et al. 2002). Mode of action of benzimidazoles affects mitotic processes and cytoskeletal development (Davidse 1986). Because the mode of action of both groups is highly specific, the risk of pathogen resistance is generally very high; and really resistance of *V. inaequalis* to both of groups was developed within two years of intensive use. While benzimidazoles are not registered for protection against the scab in the Czech Republic, presently strobilurins are registered for wide use in many crops. In contrast, fungicides with more than one target site in the fungal cell have been used for more than 30 years before the resistant isolates have been found (Deising et al. 2008). Key *cytb* gene mutation G143A causing very high resistance of *V. inaequalis* to strobilurins described Zheng et al. (2000). Mutant allele E198A of the beta-tubulin (*beta-tub*) gene linked to high level of resistance to benomyl identified Koenraadt et al. (1992). The speed of mutants' propagation depends on the pathogen biology as well as the number of fungicide applications per season (Sierotski et al. 2000; Barr et al. 2005; Lesemann et al. 2006; Chen et al. 2007). Last research objectives lead to developing of different detection techniques for monitoring of causal mutations (Michalecka et al. 2011).

The main objective of this study was to evaluate frequency of G143A and E198A mutations, to bring the information about dispersion of mutants in orchards in the Czech Republic and inform about potential risk of strobilurins use in apple growing systems.

MATERIAL AND METHODS

Obtaining and cultivation of *V. inaequalis* isolates. In total 136 cultures of *V. inaequalis* from 26 localities in the Czech Republic were collected and analysed in years 2007–2011. Isolates were collected in both scab resistant cultivars affected by pathogen scab races and chemically treated orchards of scab susceptible cultivars where the strobilurins efficacy loss was repeatedly reported. Also isolates from never treated trees were obtained. Conidia from spore-forming lesions of infected leaves were transferred to Petri dish with 2% Water Agar medium (Fluka, Sigma Aldrich, Seelze, Germany) and spread on the surface by dry sterile glass stick. After 48 h incubation in 22 ± 2°C germinated conidia were found under microscope and transferred to 4% Chloramphenicol Yeast Glucose Agar (HIMEDIA, Mumbai, India) to avoid undesirable contamination. Cultures were maintained alternately on 3.9% PDA (Potato Dextrose Agar, HIMEDIA) and 4% Malt Extract Agar (HIMEDIA).

Test of conidia germination and mycelial growth in kresoxim-methyl. Conidia germination was tested in 80 ml drops of 0.02% (w/v) solution of kresoxim-methyl (KM) fungicide Discus (BASF, Ludwigshafen, Germany). Rate of spores' germination in drops of tap water was used as control. Conidial suspensions were incubated at the dip slides at 22 ± 2°C for 48 h in the daylight. After incubation 500 of conidia were counted per sample. Each conidium producing the germ 1.5 times longer than the length of a spore was evaluated as germinating. For each sample the Relative Conidia Germination (RCG) was calculated. RCG = % of germinating conidia in KM × 100/% of germinating conidia in tap water.

In mycelial growth assay 20 monosporic mycelial plugs (2–3 mm^2) of each isolate were transferred from seven days old PDA culture to PDA plates supplemented with 0.2 g/l of the Discus and simultaneously on control plates without fungicide supplement. Fungicide was added after PDA sterilization into fluid medium at 40°C. Plates were incubated for 21 days at 22 ± 2°C in the daylight. The

Table 1. Parameters of used primers

Gene	Primer	Sequence 5'–3'	T_m (°C)	Position in gene	Length (bp)	Final analysis
cytb	VB4F VB4R	tgcaagataaatctgagttgacg tgttgttaggctcttcaatgaataat	58	5390–5547 exon 4	158	SSCP
beta-tub	T6IIF T6IIR	ctccaagattcgcgagga tggataagattgttagcaggtgtg	58	1218–1767 exon 6	550	*Bst*UI

SSCP – ssDNA conformation polymorphism detecting method

Relative Mycelial Growth (RMG) was calculated. RMG = % of mycelial growth in KM × 100/% of mycelial growth on plates without KM.

DNA extraction and amplification of target genes' regions. DNA was extracted from fungal mycelium by GenElute Plant Mini Kit (Sigma Aldrich, Seelze, Germany). Up to 100 mg of mycelium was homogenised in liquid nitrogene (LN) in 2 ml polypropylene tube by glass stick. The LN was added directly into tube for optimal disruptions of mycelium. The homogenate was extracted using standard plant miniprep protocol with two-times prolonged step of the lysis. DNA was eluted into two 75 ml portions of TE buffer. Quality of DNA sample was evaluated by NanoPhotometer (Implen, München, Germany).

Primer pairs flanking key parts of target genes were designed by means of NCBI Primer-BLAST (Ye et al. 2012) using sequences of *cytb* gene (NCBI entry AF004559 by Zheng et al. 1997) and *beta-tub* gene (NCBI entry M97951 by Koenraadt et al. 1992). Information about primers are summarised in Table 1. Polymerase chain reaction (PCR) mixture (25 ml) contained 40 ng of DNA, 1× *Taq* + KCl PCR reaction buffer (Fermentas, Vilnius, Lithuania), $MgCl_2$ (2.5 nmol/ml), equimolar mixture of dNTP (0.25 nmol/ml), primer pair (0.3 nmol/ml) and 0.5 unit of *Taq* DNA polymerase (Fermentas). Amplification conditions were optimised as follows: 1× 95°C 3 min followed by 35 cycles consisted from denaturation (95°C, 30 s), annealing (58°C, 30 s) and extension (72°C, 60 s). Amplification was finished by final extension (72°C, 5 min).

Detection of cytb-G143A and beta-tub E198A mutations. The method detecting ssDNA conformation polymorphism (SSCP) performed in DCodeTM (Bio-Rad, Hercules, USA) was used for detection G143A. Conditions for SSCP analysis were as follows. The 16 × 16 cm gel consisted of 8% acrylamide-bisacrylamide mixture (37.5:1), 0.5× TBE buffer and 10% of glycerine. 10 ml of PCR products were mixed with 10 ml SSCP Loading Dye (Bio-Rad), denaturised for 10 min at 94°C and immediately moved on the ice. After 20 min of cooling the samples were loaded on the gels and separated for 3 hours. Power supply was set on 30 W. Initial temperature of separation system was 5°C and after start of separation the temperature was stabilised by ice bath at 10°C. After the separation, gels were stained by ethidiumbromide for 15 min and then visualised and documented by GelDocXR (Bio-Rad). Staining bath consisted of 0.5 l of electrophoretic buffer supplemented by 0.8 mg of ethidiumbromide.

T6II amplicons were analysed by *Bst*UI restrictase (Fermentas) with respect to manufacturer's recommendation. Polymorphisms were detected by electrophoresis in 2% agarose gel stained by ethidiumbromide (0.5 mg/ml).

RESULTS AND DISCUSSION

Spores germination and mycelial growth under kresoxim-methyl

Set of 78 isolates was evaluated by conidia germination test. Mycelial growth was evaluated for 54 isolates. Results showed high rate of KM resistance (Table 2) in evaluated isolates. The control isolate Lázně Bělohrad obtained from the solitary tree never treated by fungicides demonstrated RCG = 0 whilst isolates from orchards periodically treated by strobilurins were highly resistant (RCG varied from 30 to 96%). Already 15% RCG demonstrates high risk for economically significant disease development. Similar situation was observed in tests of relative mycelial growth (RMG). In isolates Choustníkovo Hradiště, Kamenice (cv. Golden Delicious) and Břasy RMG equal to 100% was observed. The test is well informative in actual status of resistance in orchard and can be good tool to verify of resistance in cases when the chemical protection failed. But there was also repeatedly observed low value of RCG of isolate Rohozec (cv. Šampion) in relation to presence of G143A. This discrepancy can be partially explained by finding of Zheng et al. (2000). They studied mutation ratio of the *cytb* gene in laboratory conditions and results indicated very low value of this parameter (about 4.4×10^{-8}). If the mutation ratio in natural conditions is similar, molecular analysis can detect hidden mutation in isolate and very low RCG is then observed due to low abundance of mutated spores in the sample. It is possible believe that the test of conidia germination is not fully sufficient to monitoring of resistant populations in orchards and it should be supported by direct molecular screening of population. This presumption corresponds to results of Fontaine et al. (2008).

Molecular detection of cytb and beta-tub mutations

Primers VB4 amplified fragment 158 bp including the exon 4 of *cytb* in accordance with expected

Table 2. Characterization of *Venturia inaequalis* isolates

Locality	Cultivar	G143A freq.	E198A freq.	RCG (%)	RMG (%)
Bílé Podolí	Golden Delicious	1.0	1.0	59	n
Brno	Golden Delicious	0.0	0.0	31	93
Břasy	Unk.	1.0	1.0	30	100
Dobrá Voda	Jonagold	1.0	0.0	62	96
Dolany	Golden Delicious	1.0	1.0	37	85
Holovousy "Včelín"	Idared	1.0	0.86	88	100
Holovousy "Školka"	TSR33T239	1.0	1.0	n	n
Holovousy "Kamenec"	Gloster	1.0	1.0	96	94
Choustníkovo Hradiště	Jonagold	1.0	0.5	91	100
Kamenice	Golden Delicious	1.0	0.5	67	100
Kamenice	Šampion	1.0	0.0	72	76
Kladno "Štěpánská"	Unk.	0.0	0.5	n	n
Kladno "Sítná"	Unk.	0.0	0.0	n	n
Kopidlno	Otava	0.0	0.0	n	n
Lázně Bělohrad	Unk.	0.0	0.5	0	0
Lomnice	Rubinola	0.0	0.0	n	n
Lomnice	Šampion	0.0	0.0	n	n
Lomnice	Selena	0.0	0.0	n	n
Lysice "Agrokonzor"	Golden Delicious	1.0	1.0	n	n
Lysice	Topaz	0.0	0.0	n	n
Lysice	Vanda	0.0	0.0	n	n
Lysice	Jonagold	1.0	1.0	40	100
Lysice	Topaz	1.0	0.0	n	n
Petrovičky	Golden Delicious	1.0	0.66	41	n
Praha Ruzyně	Rubín	1.0	1.0	52	84
Radňov	Panenské	0.0	0.0	n	n
Rohozec	Šampion	1.0	0.5	3	n
Rohozec	Idared	1.0	1.0	n	n
Rohozec	OR45T132	0.0	0.0	n	n
Rohozec	Prima	0.0	0.0	n	n
Rohozec "Brťov-Jeneč"	Goldstar	0.0	0.5	n	n
Rohozec "Brťov-Jeneč"	Rubinola	0.5	0.0	n	n
Starý Lískovec	Unk.	1.0	1.0	31	n
Těšetice	Golden Delicious	1.0	0.58	34	n
Tišnov	Selena	1.0	0.0	n	n
Tišnov	Topaz	1.0	0.0	n	n
Tuchoraz	Golden Delicious	1.0	1.0	95	97
Velké Bílovice	Golden Delicious	1.0	1.0	59	92
Velké Němčice	Idared	1.0	1.0	n	n
Velký Třebešov	Golden Delicious	1.0	1.0	n	n
Veselý Žďár	Rubín	0.0	0.66	n	n
Žernov	Topaz	1.0	1.0	n	n

Unk. – unknown genotype; n – not evaluated; RCG – relative conidia germination; RMG – relative mycelial growth

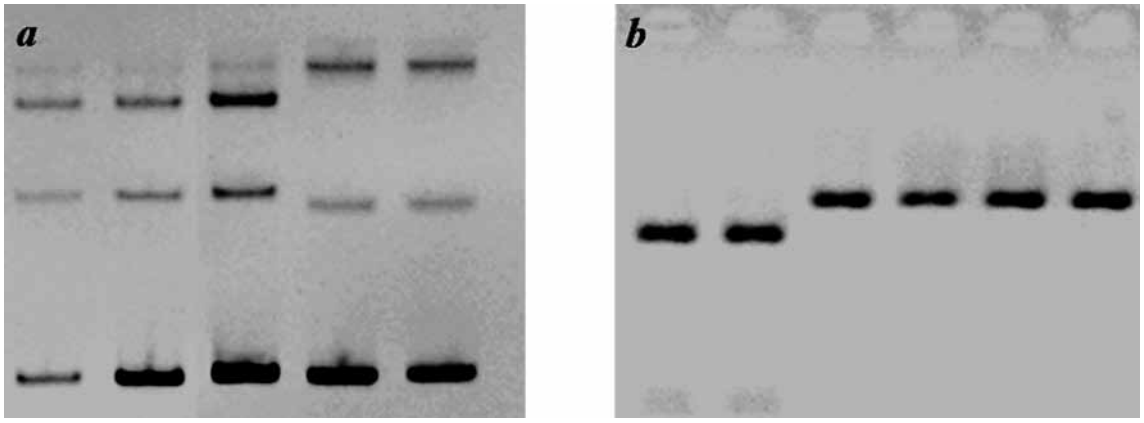

Fig. 1. Electrophoreograms
(a) lanes from the left: 1–3 – mutation G143A; 4–5 wild type allele *cytb*., (b) lanes from the left: 1–2 – mutation E198A; 3–6 wild type allele *beta-tub*

length. Amplicons from KM-resistant and KM-sensitive *V. inaequalis* strains were sequenced with the aim to verify the amplicon identity and causality of mutation to resistance of our isolates. The G/C substitution was found in the nucleotide 77 of the amplicon typical for KM-highly resistant strains. Results corresponded to Zheng and Köller (1997) because presence of mutation is in the typical point (G143A). Others mutations described in cited work and causing partial resistance were not detected in our samples. It can be explained by rare occurrence of these mutations or by their low potential to express the resistance and also due to low selection pressure of fungicide. Optimised SSCP analysis detected differences in relative electrophoretic mobility (REM) of individual ssDNA molecules in patterns of KM-resistant and KM-sensitive strains (Fig. 1). Although this method was developed about 20 years ago it is used in many applications as reported Hayashi (1992) or Ortí et al. (1997). The main advantage of the method is simplicity and cheapness (it does not need expensive laboratory equipment) so it is suited also for small laboratories. Credibility of the method was tested in all samples in absolute concordance of SSCP dimorphism and nucleotide sequence of amplicon. Association of G143A distribution and results of both fungicide tests were evaluated. The critical value of RCG was set at 15%. Results of germination test and presence of G143A highly associated (association coefficient Q = 0.9562). The correlation coefficient (r = 0.5259) was found as highly significant at significance value α = 0.01. Association of molecular results and mycelial growth in KM positive plates was similar (Q = 1.0 and r = 0.8854). Isolate Brno didn't show presence of the G143A and simultaneously it was evaluated as resistant in the both of fungicide tests. Zheng et al. (2000) observed this in several cases and identified an alternative pathway to mitochondrial respiratory chain. This is probably the reason of our finding because of relatively low RCG of the isolate when conidia can be more susceptible.

Detection of E198A mutation of *beta-tub* gene brought expected results. Primer set T6II amplified DNA fragment in length 550 bp in all evaluated genotypes and *Bst*UI restrictase assay detected two alleles of this marker in isolates collection. Cleavable marker provided two restriction fragments in length 412 bp and 138 bp and it should be associated to benomyl resistance (Koenraadt et al. 1992). In our work resistance to benomyl was not evaluated. Descriptive results of molecular analyses Table 2, Figs 1 and 2 certify.

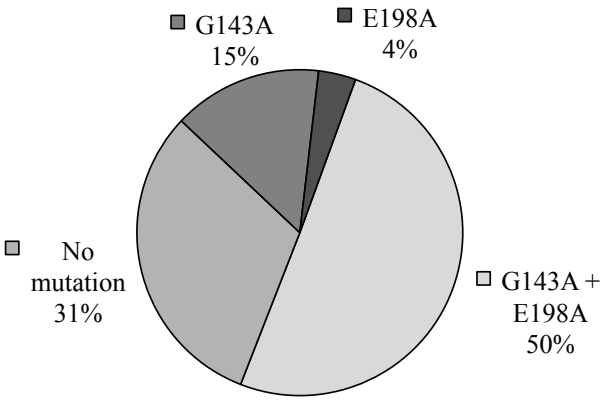

Fig. 2. Distribution of mutation within isolates

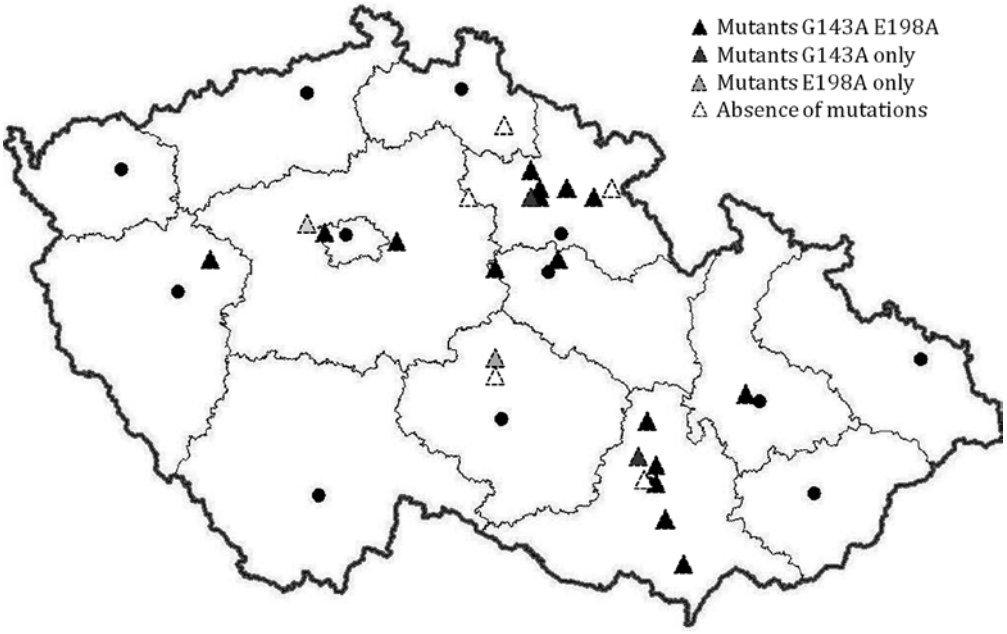

Fig. 3. Distribution of G143A and E198A mutants within 2007–2011

Occurrence of mutations in the Czech Republic

In 31% of isolates were not detected any mutations and conversely 50% isolates accumulated both mutations. It is important that G143A substitution was noted in 65% isolates while the E198A was detected in 54% of isolates (Fig. 2). Map (Fig. 3) informs about distribution of resistant strains in the Czech Republic. It is evident, that mutants are spread out in all important apple growing regions. Information about specific locations are summarised in Table 2. What is the practical impact of this finding? In the light of current status and genetic background of strobilurin resistance in orchards, pure strobilurin fungicides should be completely removed from protection against *V. inaequalis*. With the goal to decrease resistance risk or delay its development the use of strobilurins in tank-mixes is recommended in combination with contact active substances with different and/or multisite action mode. Using of strobilurins in orchards where a resistant strain of pathogen is occurring definitely lacks any effectiveness. Qo inhibitors KM, trifloxystrobin and azoxystrobin occupy practically 36% of registered active substances in Czech register specified for regulation of the apple scab, but the really used amount in total volume of fungicides applied for protection of fruits is not so significant because in the long time period the consumption of Qo inhibitors is up to 1% of total fungicides consumption. These active substances have a good ability to prevent spore germination and also they are very selective and friendly to the environment. So each individual grower using strobilurins should evaluate the economy of treatment in connection to current fungicide efficacy in his orchard.

The scientific objective of this work was to evaluate the potential of *V. inaequalis* populations in the Czech Republic to loss of sensitivity to strobilurin fungicides. Results of molecular analyses indicate very high abundance of *cytb* G143A causing resistance of pathogen to strobilurins. Comparing to the frequency of *beta-tub* E198A substitution causing the resistance to benomyl non-used in protection in orchards for more than 20 years due to its efficacy loss the current frequency of G143A is just higher about 9%. For this high risk of rapid spreading of *cytb* mutants within *V. inaequalis* population exists. It means that in similar cases the use of pure strobilurins cannot be recommended at the present time and in a future as well, because the frequency of mutation can be probably highly fixed for long time period similarly to the situation about of mutation E198A.

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