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# Characterisation of the first *Stemphylium vesicarium* isolates resistant to strobilurins in Italian pear orchards

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**Abstract** Up to 2005 the sensitivity of *Stemphylium vesicarium* (Wallr.) Simm., the causal agent of pear brown spot, to the strobilurin fungicides kresoxim-methyl, trifloxystrobin and pyraclostrobin was still comparable with baseline values associated with good efficacy in the field. During 2006, the first resistant isolates were detected in two commercial pear orchards in the Emilia-Romagna region (Italy), one of which was affected by considerable control failure linked to strobilurin treatments as demonstrated in a field trial. In vitro sensitivity tests with 0.5 mg $l^{-1}$ of kresoxim-methyl, trifloxystrobin and pyraclostrobin showed that in the population collected in the orchard with control failure the conidial germination was greater than 90% compared to an untreated control both in 2006 and in 2007, i.e. 1 year after the suspension of strobilurin applications. In the other orchard, where only a few symptomatic fruits were found and the strobilurins were still in use, the conidial germination was lower, about 50% in 2006 and 25% in 2007. The molecular analysis of mitochondrial cytochrome b gene of some monospore isolates with different levels of sensitivity confirmed the presence of the mutation causing G143A substitution in all the resistant isolates. In conclusion, both in vitro tests and molecular analysis confirmed the first occurrence of *Stemphylium vesicarium* resistance to all strobilurin fungicides tested.

**Keywords** Brown spot · Fungicide resistance · Kresoxim-methyl · Pyraclostrobin · Trifloxystrobin

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For 9 years since their introduction in pear disease control in Italy (kresoxim-methyl in 1998, trifloxystrobin in 2002 and pyraclostrobin in 2006), strobilurin fungicides have been very interesting compounds in the control of brown spot, the main fungal disease on pear in Italy. Their efficacy against this pathogen is indeed still generally comparable to baseline values (Alberoni et al. 2008a). This behaviour is very different from what has been observed with other pathogens, where resistance problems arose after a few years of field application (Heaney et al. 2000; Ishii et al. 2001; Kim et al. 2003; Ma et al. 2003). This aspect is quite important because the control of pear brown spot needs a considerable number of fungicide treatments, from petal fall to fruit ripening (Brunelli et al. 2004), and a wide range of fungicides with different mechanisms of action is the basis of the anti-resistance strategy. *Stemphylium vesicarium*, the agent of pear brown spot, has already developed resistance toward dicarboximide fungicides (Brunelli et al. 1997) and in a few cases also toward fludioxonil

(Alberoni et al. 2008a). These fungicides, as well as dithiocarbamates, captan, tebuconazole, strobilurins and boscalid, are key-products in spray schedules. Dicarboximide resistance is qualitative, and when it occurs the compounds are thus no longer active and heavy yield losses (up to 90%) are recorded. In these cases the use of dicarboximides therefore has to be suspended and they are often replaced with strobilurins. Unfortunately, the latter have a high risk of resistance and it is thus important to avoid overuse of these fungicides. After many years of applications against pear brown spot, the efficacy of strobilurins in the field is still good; indeed, just a few resistant isolates were found for the first time only in 2006 after a particularly intense use of kresoxim-methyl and trifloxystrobin for several years (Collina et al. 2007).

The aim of this study was to characterise the first populations of *S. vesicarium* with strobilurin-resistant isolates, collected in two orchards of Abbé Fétel for two consecutive years (2006 and 2007) within an extensive monitoring study in the Po valley on *S. vesicarium* sensitivity to fungicides.

In the first location (origin of population Sv293), after 7 years with 5–6 treatments with kresoxim–methyl or trifloxystrobin, extensive symptoms appeared in 2006 after repeated strobilurin applications. The disease incidence on fruits increased up to 80% at the end of the season and after a total of 6 strobilurin treatments. In 2007 their use was suspended in the commercial orchard but an experimental trial was carried out with various fungicides normally used against pear brown spot to fully confirm that the yield losses were linked to strobilurin treatments. In fact, the plots sprayed with kresoxim-methyl or trifloxystrobin showed a disease incidence quite similar to the untreated plots and thus a very poor control compared to the other fungicides (Alberoni et al. 2008b). In the second orchard (origin of population Sv393), no control failures were noted with the use of these compounds in 2006 and in 2007, even if they had been used for 5 years with 3–4 treatments per year. In both years about 20 symptomatic pears were collected in the first orchard while only 4 symptomatic fruits were found in the second orchard because of the low disease incidence. *S. vesicarium* colonies were isolated from each single spot of each fruit on V8 juice agar amended with 50 mg $l^{-1}$ of streptomycin sulphate according to the methodology reported by Alberoni et al. (2005). The populations considered in the assays were obtained by mixing the spores of all the respective *S. vesicarium* colonies. Each colony was also tested separately to confirm the results on populations of monospore isolates obtained from colonies. The reference-sensitive population belonged to our collection of fungi.

In vitro sensitivity assays on conidial germination were carried out on 1.5% water agar with active ingredients of kresoxim-methyl, trifloxystrobin and pyraclostrobin (Sigma-Aldrich, St. Louis, USA) at the concentration of 0.5 mg $l^{-1}$ according to the methodology reported by Collina et al. (2007); the evaluations were made 3, 5 and 24 h after inoculation. The results are expressed as the percentage of conidial germination compared to an untreated control.

After just 3 h of incubation, the population Sv293 showed a germination greater than 90% of the untreated control at 0.5 mg $l^{-1}$ of the three tested strobilurins, both in 2006 and in 2007 (Table 1). The analysis of all the colonies forming the population showed only 1 colony out of 13 with no germination at the discriminatory concentration in 2007, which was therefore considered sensitive to strobilurins (Fig. 1). All the other colonies had germination ratios greater than 72%, thus as resistant as the original population, just like all the colonies obtained in 2006. The monospore isolates of two colonies (6,13) showed a sensitivity comparable to their original colonies (Fig. 1). The other population (Sv393) showed a lower germination (about 50% of the untreated control in 2006 and under 37% in 2007) within 3 and 24 h of incubation (Table 1). This behaviour could be explained by the observation of the analysis of the colonies forming the population; indeed they had a considerably different level of sensitivity. In 2006 two colonies were resistant, with a germination similar to the untreated control, while two colonies were sensitive, with no germination at the discriminatory concentration for all the tested strobilurins. A similar behaviour was also observed in 2007; three colonies were sensitive and one was resistant (Fig. 2). In this case too, the monospore isolates of two colonies (1,2) showed a sensitivity comparable to their original colonies in both years.

Finally, the total DNAs of some single spore isolates obtained from the colonies of each population selected for their different levels of sensitivity in in vitro tests were extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

**Table 1** Activity of kresoxim-methyl, trifloxystrobin and pyraclostrobin against conidial germination of *S. vesicarium* populations collected in two orchards (Sv293 from an orchard with yield losses and Sv393 from an orchard without disease control problems) for two consecutive years (2006–2007) compared to a reference-sensitive population (SvS)

| Hours of incubation | Population ID | % of conidial germination at 0.5 mg $l^{-1}$ compared to an untreated control | | | | | |
|---|---|---|---|---|---|---|---|
| | | kresoxim-methyl | | trifloxystrobin | | pyraclostrobin | |
| | | 2006 | 2007 | 2006 | 2007 | 2006 | 2007 |
| 3 | Sv293 | 90 | 99 | 93 | 94 | 93 | 90 |
| | Sv393 | 46 | 15 | 49 | 28 | 49 | 21 |
| | SvS | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Sv293 | 97 | 99 | 96 | 94 | 95 | 91 |
| | Sv393 | 67 | 15 | 50 | 28 | 49 | 22 |
| | SvS | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | Sv293 | 100 | 99 | 96 | 99 | 96 | 97 |
| | Sv393 | 68 | 22 | 50 | 37 | 52 | 32 |
| | SvS | 0 | 0 | 0 | 0 | 0 | 0 |

Two PCR primers, RSCBF1 (5′-TAT TAT GAG AGA TGT AAA TAA TGG-3′) and RSCBR2 (5′-AAC AAT ATC TTG TCC AAT TCA TGG-3′), designed by Fraaije B.A. (Ishii et al. 2001), and two specific primers, SVCBFw (5′-AGT GAA TAC GGT TAA AGA AGC-3′) and SVCBRe (5′-CCT ATC GTA GGT AGT AAA CTA-3′), designed in *S. vesicarium* cytochrome b around the G143A mutation site were used. PCR amplifications were performed with 1 µl total DNA in a Personal Cycler (Biometra, Gottingen, Germany) in 50 µl reaction mixture containing 5 µl 10X Ex Taq™ Buffer, 0.2 µM primers, 0.2 mM of each dNTP and 1.25 U TaKaRa Ex Taq™ (TaKaRa, Shiga, Japan).

The initial denaturation of the thermal cycle was 95°C for 3 min and it was followed by 40 cycles at

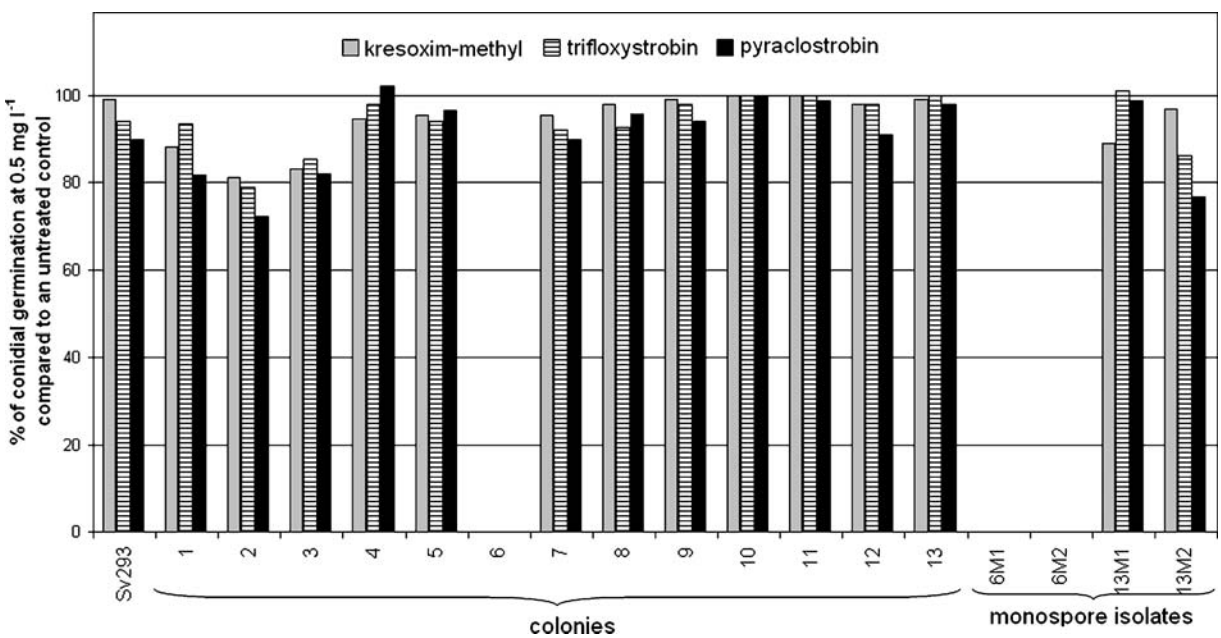


**Fig. 1** Sensitivity to strobilurins of *S. vesicarium* population Sv293 in 2007, 13 colonies and some monospore isolates

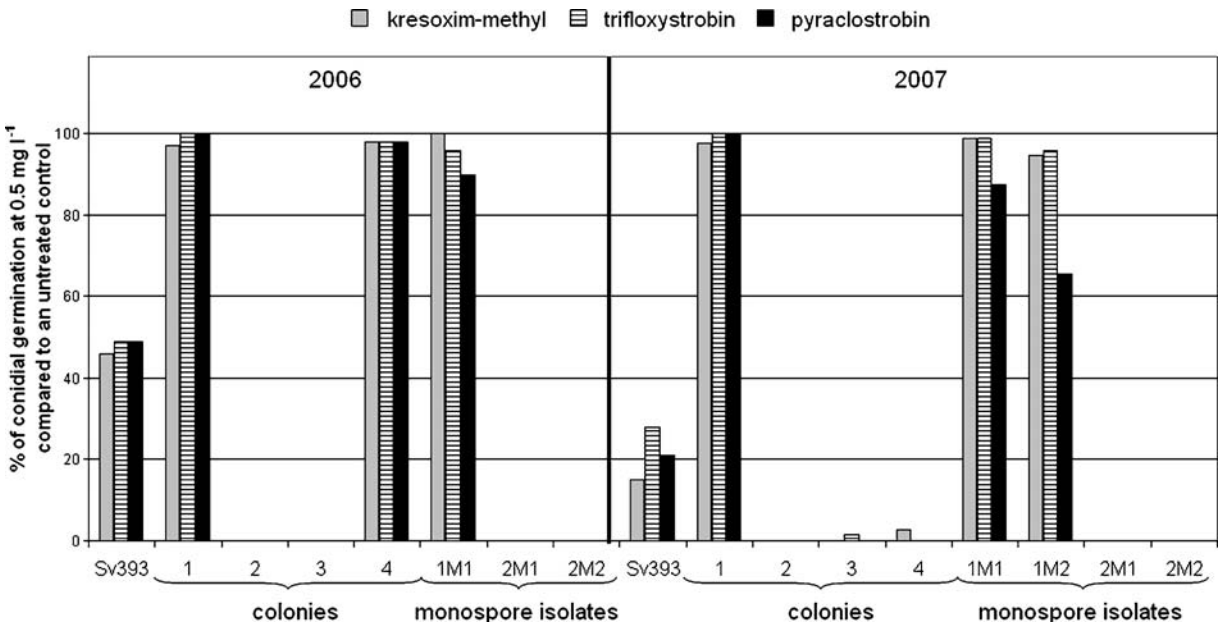


**Fig. 2** Sensitivity to strobilurins of *S. vesicarium* population Sv393 in 2006 and 2007, 4 colonies and some monospore isolates

94°C for 40 s, 50°C or 58°C (respectively for the two pairs of primers) for 40 s and 72°C for 1 min 30 s or 40 s respectively. The final extension was for 10 min at 72°C. PCR products were separated through electrophoresis in 0.5X TBE buffer on 1.5% agarose gel and stained with ethidium bromide. Sizes of DNA fragments were determined by comparison with 100 bp DNA ladder (New England BioLabs, Ipswitch, USA). PCR products were then purified using a GeneElute PCR Clean-up kit (Sigma-Aldrich, St. Louis, USA) and sequenced by BMR Genomics, University of Padua, Italy (http://www.bmr-genomics.it). DNA sequences from each isolate were translated into amino acid sequences using mold mitochondrial code by the computer program Transeq (http://bioweb.pasteur.fr/seqanal/interfaces/transeq.html).

This molecular analysis of some monospore isolates of the tested colonies showed that all the resistant isolates had an alanine in position 143 of the cytochrome b aminoacidic chain, unlike the sensitive monospore isolates that conserved the glycine. The substitution G143A is therefore correlated with strobilurin resistance also in *S. vesicarium* as already observed in other pathogens (Heaney et al. 2000; Ishii et al. 2001; Kim et al. 2003; Ma et al. 2003; Koller et al. 2004), as it is the most common substitution linked to strobilurin resistance.

The results obtained in this study confirmed the first occurrence of *S. vesicarium* resistance to strobilurins in the field (Collina et al. 2007). In the case where the population had a germination comparable to the untreated control (which means that almost all the colonies were resistant), failure occurred in the field, as confirmed by the fungicide efficacy trial. These observations, in accordance with the presence of the G143A substitution, confirm that this was the first case of practical resistance of *S. vesicarium* to strobilurins. On the contrary, more samples are needed in the future to confirm that a population with a germination lower than 50% of the untreated control does not cause problems in disease control even if the strobilurins were still used, as observed for the other population that was based only on 4 colonies, due to a very low incidence of the disease in the orchard.

In conclusion, *S. vesicarium* is able to develop resistance to strobilurins although after many years of intense use of these compounds (7 years with 5–6 treatments). The first cases appeared, in fact, 8 years after the introduction of these compounds in Italy on pear and with a high percentage of resistant isolates. When resistant isolates were selected, they persisted at least 1 year after suspension of strobilurin applications, although in the 2007 analysis one colony out of thirteen was found to be sensitive.

Further studies are therefore needed to better clarify the correlation between the frequency of resistant isolates and strobilurin efficacy in the field and other characteristics of *S. vesicarium* resistance.

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