

## Sensitivity of *Septoria pyricola* isolates to carbendazim, DMI and QoI based fungicides and to boscalid, in Greece

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**Summary.** The sensitivity of 36 *Septoria pyricola* single spore isolates, obtained from isolated pear orchards, to carbendazim, bitertanol, flusilazole, myclobutanil, azoxystrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin and boscalid, was studied *in vitro*. Spore suspensions were point-inoculated on media amended with various concentrations of fungicides and the minimum inhibitory concentration (MICs) of the fungicides was determined. Most isolates were highly resistant to carbendazim, forming colonies even at concentrations of 100 mg L<sup>-1</sup> of the fungicide. A few isolates failed to form colonies with carbendazim concentrations of 0.1 and 10 mg L<sup>-1</sup>; these isolates were designated carbendazim-sensitive and moderately carbendazim-resistant respectively. The MIC of DMI fungicides was up to 1 mg L<sup>-1</sup> with some isolates, and the mean MICs of bitertanol, flusilazole and myclobutanil were 0.133±0.036, 0.075±0.044 and 0.230±0.038 mg L<sup>-1</sup> respectively. The MIC of the QoI fungicides was up to 0.1 mg L<sup>-1</sup> with most isolates, but some isolates were 100-fold less sensitive to azoxystrobin. The mean MICs of azoxystrobin, kresoxim-methyl, pyraclostrobin and trifloxystrobin were 0.177±0.040, 0.075±0.035, 0.067±0.063, and 0.073±0.065 mg L<sup>-1</sup> respectively. Overall, the MIC of boscalid was 1 mg L<sup>-1</sup>, and the mean MIC was 0.111±0.044 mg L<sup>-1</sup>. The ED<sub>50</sub> values of representative isolates are also presented, as determined by colony formation with dispersed spore inoculation on a medium amended with fungicides. The results show that the benzimidazoles are ineffective against *S. pyricola* isolates in Greece and suggest that the future effectiveness of the DMIs is at risk.

**Key words:** leaf fleck, septoriosiis, pears, fungicide resistance.

### Introduction

*Septoria pyricola* (Desm.) Desm., the anamorph of *Mycosphaerella pyri* (Auersw.) Boerema, is a widespread pathogen that causes leaf fleck disease of pear, also called leaf spot or septoriosiis (Figure 1a, b). Leaf fleck, although rarely reported, causes superficial lesions, reducing fruit quality (Figure 1c). Flecked or spotted fruits cannot be sold as high grade and are liable to further infection from rot micro-organisms during storage (Sivane-

san, 1990; Aloj *et al.*, 1994). Ascospores released by fallen leaves provide the primary inoculum for infections in early spring. Leaf fleck is favoured by relatively high temperatures (infections start at 20°C) and rainy weather (Gort *et al.*, 2000). In late spring and summer the disease progresses rapidly through continuous infection caused by pycnidiospores produced on the spotted leaves (Tzavella-Klonari and Tamoutseli, 1986; Sivanesan, 1990). Although leaf fleck of pear is very common in Mediterranean countries, it was until recently considered to be only of minor importance, and regular fungicide applications, intended primarily for the control of scab (*Venturia pirina*) were thought to eliminate *Septoria* as well. In recent years, howev-

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er, outbreaks of leaf fleck have become more common in Italy on pears cv. Coscia (Aloj *et al.*, 1994), and in Greece on cv. Krystalli (Biris *et al.*, 1991; Pappas *et al.*, 2006), while scab on the contrary has remained rare. The local fresh pear cultivar Krystalli is widely grown in Greece because it is moderately resistant to fireblight caused by *Erwinia amylovora*. According to Gort *et al.* (2000), other more popular pear cultivars such as Passa Crassana and Williams also suffer from serious *Septoria* attacks in Spain when weather conditions are favourable.

Over the last four decades, widespread and intensive spraying of the benzimidazoles and sterol demethylation inhibitors (DMIs) on apples and pears has led to the early appearance of fungicide resistance in scab pathogens in a number of countries (Shabi and Ben-Yephet, 1976; Gilpatrick, 1982; Stanis and Jones, 1985; Köller and Scheinpflug, 1987). Since 1996, the strobilurins (quinone outside inhibitors, QoIs) **have been applied** against fungal diseases of various crops, and resistance against these agents arose early and is now well documented in many plant pathogens (Bartlett *et al.*, 2002). Two members of the

QoI class, azoxystrobin and kresoxim-methyl, the first to protect vegetables, the second to protect pears and other crops, were registered in Greece in 2002. This was followed two years later by trifloxystrobin (for pears and other crops) and, more recently, by formulations containing mixtures of pyraclostrobin plus boscalid (in 2006 for vegetables, in 2008 for pome fruit).

Hitherto, however, not much information was available about the sensitivity of *S. pyricola* to all these fungicides. In 2006, nine out of ten isolates were found to be highly resistant *in vitro* to the benzimidazole fungicide carbendazim (methyl benzimidazol-2-ylcarbamate, mbc). **All these isolates**, irrespective of their sensitivity to carbendazim, were however highly sensitive to bitertanol (a DMI fungicide) and to the QoI fungicide kresoxim-methyl (Pappas *et al.* 2006). In addition, benomyl at an early date (Biris *et al.*, 1991), and more recently carbendazim (Pappas *et al.* 2006), have lost their capacity to control leaf fleck in naturally infected pear orchards. Moreover, Chatzidimopoulos and Pappas (2008) reported that when a 1:1 mixture of a carbendazim-sensitive and a carbendazim-resistant isolate was mechanically inocu-

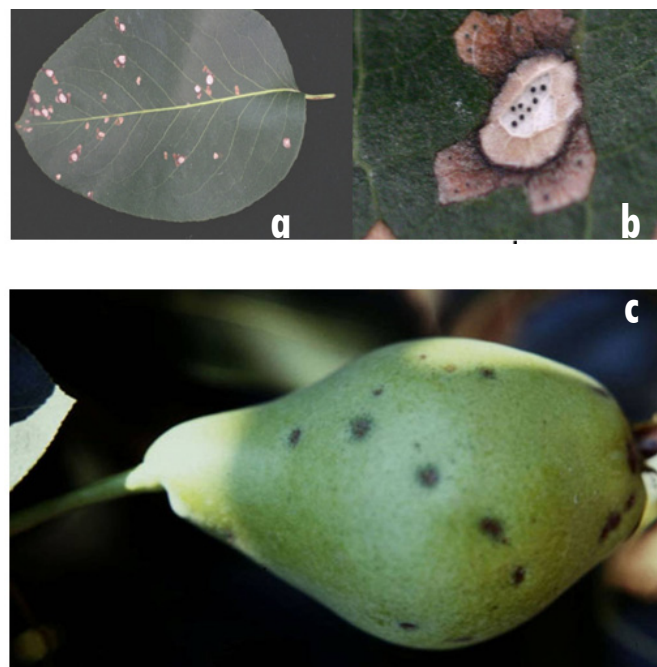


Figure 1. Disease symptoms of *Septoria pyricola* on pear: a, spots on leaves; b, pycnidia ( $\times 10$ ) on a leaf spot; c, lesions on fruit.

lated, the infection on carbendazim-treated trees was as severe as that in the controls. In contrast, these researchers found that the DMI fungicides bitertanol and flusilazole, the QoI fungicides azoxystrobin, kresoxim-methyl, trifloxystrobin and pyraclostrobin plus boscalid, completely controlled *S. pyricola*.

The purpose of this work was to evaluate the range of sensitivity of *S. pyricola* isolates to carbendazim and to members of the DMI class, which have been applied for more than four decades to control both scab and leaf fleck on pears. Representative fungicides of the recently introduced QoI class were also tested, in order to provide reference sensitivity data of *S. pyricola* populations prior to the intensive application of this class of fungicides. The intrinsic activity of a new specific fungicide, boscalid, a succinate dehydrogenase inhibitor (SDHI), was also studied in order to pro-

vide baseline sensitivity data of the *S. pyricola* populations, prior to its registration and introduction into agricultural practice in Greece.

## Materials and methods

### Fungicides and chemicals

The following commercial fungicide formulations were supplied by their respective manufacturers and used in all tests: carbendazim as Carbendazim 50% a.i. WP (Cequisa SA, Barcelona, Spain), bitertanol as Baycor 25% a.i. WP (Bayer CropScience, Monheim, Germany), flusilazole as Punch 40% a.i. EC (E.I. Du Pont de Nemours & Co Inc., El paso, TX, USA), myclobutanil as Systhane 12.5% a.i. EC (Dow Agrosciences, Indianapolis, IN, USA), azoxystrobin as Ortiva 25% a.i. SC (Syngenta, Monthey, Switzerland), kresoxim-

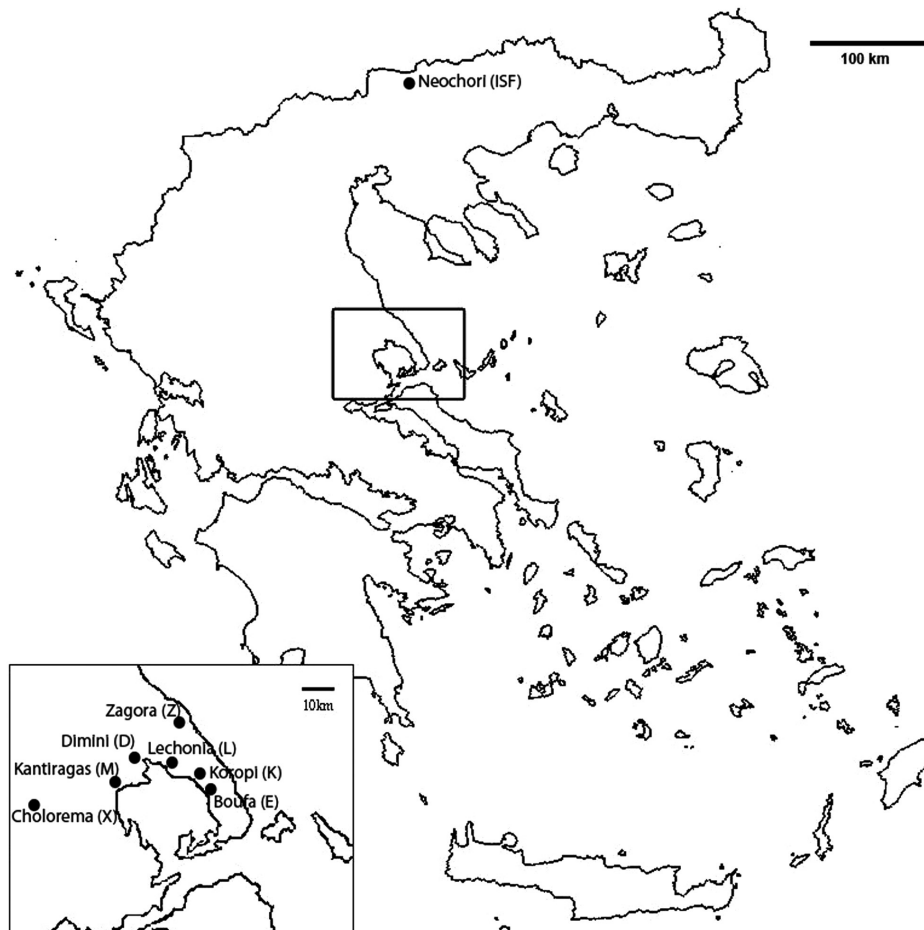


Figure 2. Map showing the sites where pear leaves spotted by *Septoria pyricola* were sampled.

methyl as Strobry 50% a.i. WG (BASF, Ludwigshafen, Germany), pyraclostrobin as F500 25% a.i. EC (BASF), trifloxystrobin as Flint 50% a.i. WG (Bayer CropScience), boscalid as BAS510 50% a.i. WG (BASF). To obtain the desired concentrations (mg active ingredient per L of growth medium), aliquots were prepared from stock solutions in dimethyl sulfoxide (DMSO). The final concentration of the solvent in the growth medium was 1% (v:v).

#### Isolates

Ten pear orchards cv. Krystalli located in

eight sites in separate locations were used to sample *Septoria* spotted leaves (Figure 2). During the period 2003–2007, leaves with distinct spots bearing oozing pycnidia of *S. pyricola* were randomly collected. A cirrus from one spot per leaf was randomly picked up with a sterilized needle, and a sparse pycnidiospore suspension in distilled water was prepared and spread on water agar in Petri dishes. After 2 days of incubation at 24°C in the dark, 36 single-spore field isolates were chosen at random. Most of these isolates came from orchards with medium/heavy disease severity, that were already treated with

Table 1. Origin of *Septoria pyricola* isolates tested for their sensitivity to different fungicides *in vitro*.

Orchard code	No. of isolates tested	Year of sampling	Fungicides applied in the year of isolate sampling	Disease severity <sup>b</sup>
E	6	2007	Carbendazim, bitertanol, myclobutanil, penconazole, trifloxystrobin	Heavy
K	3	2003	Captan, carbendazim, copper oxychloride	Heavy
L	5	2003	Captan, carbendazim, chlorothalonil	Heavy
2L	3	2003	Captan, chlorothalonil, copper oxychloride, dodine, fenarimol	Light
M	1	2004	Unsprayed, abandoned and isolated garden tree	Medium
1D	1	2004	Unsprayed orchard	Medium
ISF	2	2004	Chlorothalonil, iprodione, fenarimol, mancozeb, thiophanate-methyl, thiram	Light
X	11	2003	Carbendazim, copper oxychloride, iprodione, mancozeb, fenarimol	Medium
16X	1	2004	Bitertanol, chlorothalonil, copper oxychloride, kresoxim-methyl, myclobutanil	Light
Z	3	2004	Bitertanol, captan, carbendazim, fenarimol, mancozeb, myclobutanil	Medium
37M-1 <sup>a</sup>	1	2006	Derived from M-1 field isolate after two weeks growth on trifloxystrobin 1 mg L <sup>-1</sup>	-

<sup>a</sup>Laboratory mutant.

<sup>b</sup>Disease severity was estimated on a scale from 1(light) to 3 (heavy) by counting the number of spots on a random sample of 100 mature leaves.

the fungicides to be tested (Table 1). Disease severity was estimated on a 3-point scale, where 1, light infection (up to three spots per leaf); 2, medium infection (four to nine spots); and 3, heavy infection (10 spots or more). For this purpose a sample of 100 mature leaves was collected at random from each orchard. The mean disease severity of each orchard was calculated by adding the number of leaves at each infection grade (1–3), multiplying the number of leaves in each grade by 1, 2 and 3 respectively, and dividing the sum by the total number of assessed leaves.

The isolates were transferred to potato dextrose agar (PDA; Oxoid Ltd, Hampshire, UK) in glass tubes and after 2 weeks of growth were stored at 4°C under sterilized paraffin oil until use. Under such storage conditions the viability and sporulation of all the isolates remained unaffected for 5 years. For all the tests, pycnidiospores obtained from fresh subcultures grown on PDA for 3 weeks and incubated at 24°C in the dark were used as inoculum.

#### Fungicide sensitivity assays

According to the guidelines, a sample of 20 to 50 isolates, collected at random from a distinct location, and tested against five to ten dose rates, will in most cases give a reasonable picture of the sensitivity of a fungal population to a fungicide (Russell, 2004). In our case the sensitivity profile of 36 single-spore *S. pyricola* isolates was assessed by point inoculation (Pappas, 1997) on PDA amended with one of six concentrations for each fungicide, replicated three times. Isolates were initially selected by exposing them to a wider range of fungicide concentrations. The fungicide concentrations tested were: 0.005, 0.01, 0.1, 1, 10 and 100 mg L<sup>-1</sup> a.i. for carbendazim; 0.0001, 0.001, 0.005, 0.01, 0.1 and 1 mg L<sup>-1</sup> a.i. for bitertanol, flusilazole and myclobutanil; and 0.001, 0.005, 0.01, 0.1, 1 and 10 mg L<sup>-1</sup> a.i. for azoxystrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin and boscalid. Fungicides were dissolved in DMSO and added to 200 mL of a sterilized agar medium in flat-sided medicine bottles at a temperature of approximately 45°C. The bottles were well shaken, and their contents was distributed equally to 20 Petri dishes and allowed to set. A drop (about 35 µL) of a dense pycnidiospore suspension in sterile water (about 50,000 spores per mL) of each isolate, was pipetted on

pre-marked points on the periphery of Petri dishes containing a PDA-amended medium. After 8 days of incubation at 24°C in the dark, the Petri dishes were assessed for fungal colonies visible to the naked eye. In this way the minimum inhibitory concentration (MIC) inhibiting the colony formation of each isolate at each fungicide concentration was determined. Untreated plates served as controls.

Five isolates were selected on the basis of their response to the fungicides in the point inoculation assays. Spore suspensions of the isolates were spread on PDA amended with different concentrations of six fungicides and examined for spore germination and colony growth, as compared with the controls. Isolate ISF-1 was strongly resistant to carbendazim and moderately sensitive to bitertanol and boscalid; K-5b was moderately resistant to carbendazim; M-1 and E-1, derived from an untreated tree and from a trifloxystrobin-treated orchard respectively, exhibited low sensitivity to azoxystrobin; 37M-1 was derived from field isolate M-1 after M-1 grew for two weeks on PDA amended with 1 mg L<sup>-1</sup> trifloxystrobin. Three dishes per fungicide concentration were prepared as above and inoculated by pipetting 5 drops (about 150 µL) of a sparse pycnidiospore suspension (3,000 spores per mL) on them. The inoculum was spread evenly on the PDA with a glass rod and the dishes were incubated at 24°C in the dark. The mode of conidial germination of each treatment/replicate was monitored commencing at 24 h, and continuing until 72 h after inoculation. A conidium was taken to be germinated if it produced a germ tube(s) of at least 20 µm. After eight days of incubation, the total number of mycelial colonies visible to the naked eye on each Petri dish was recorded and the ED<sub>50</sub> of each isolate/fungicide was determined.

The experiments were repeated at least twice to detect variations in the sensitivity response of the fungal isolates to the fungicides. The data shown are the means of three replicates, pooled as though they were a single experiment.

#### Data analysis

The MICs (95% confidence intervals) were calculated by recording the MIC of each fungicide required for colony growth of each isolate, replicated three times. The mean MICs±SE ( $P=0.05$ ) were calculated by measuring the frequency distributions of the total MIC range (maximum to

minimum) in 36 isolates. The ED<sub>50</sub> ( $P=0.05$ ) values were calculated by the linear regression of the probit % of the inhibition of colony formation as a function of the log of inhibitory concentrations. Data were analyzed with SPSS statistical software (SPSS, release 17.0.0, August 23, 2008, SPSS Inc., Chicago, IL, USA).

## Results

### Fungal characteristics in the absence of fungicides

When fungicide-free media were point-inoculated with the spore suspension, clear and dense mycelial colonies, about 1 cm in diameter, formed at the inoculation points after eight days. These

colonies produced pycnidia after 15 days. When a dispersed spore suspension was inoculated, germination started after 24 h and was completed after 72 h. In all the assays, only a small proportion (5–15%) of spores with short germ tubes (less than 20  $\mu\text{m}$ ) germinated within 24 h. Germination was 55–65% after 48 h, and 90–100% after 72 h. From each 2-septate conidium, one to three germ tubes emerged; usually both outer cells of the spore germinated. When the germ tubes reached a length of about 50  $\mu\text{m}$  they started to branch out, forming a dense mycelial colony 0.8 to 1.4 mm in diameter within 8 days of incubation. The total number of individual colonies visible to the naked eye ranged from 300 to 500 per Petri dish. Mature pycnidia were produced in large clusters after 15 days of

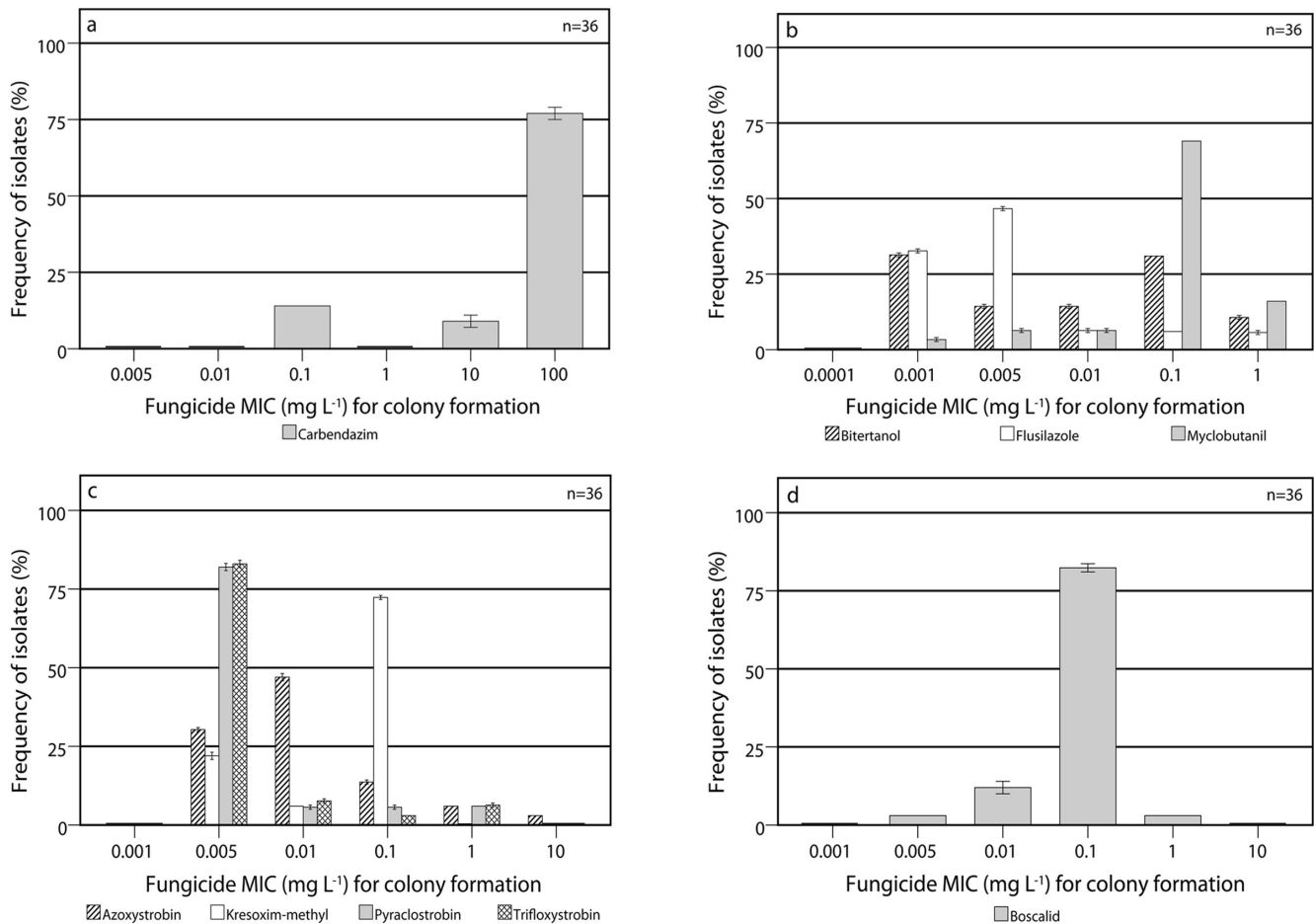


Figure 3. Frequency of sensitivity distribution for 36 *Septoria pyricola* isolates to various fungicides as studied on PDA amended medium by the point inoculation method. Bars represent 95% confidence intervals.

incubation.

#### Sensitivity of strains to carbendazim

In the point inoculation tests, 28 isolates out of 36 formed colonies at a concentration of 100 mg L<sup>-1</sup> of carbendazim. These isolates were characterized as carbendazim-resistant (mbc<sup>R</sup>). For three isolates the carbendazim MIC was 10 mg L<sup>-1</sup>, and for five isolates it was 0.1 mg L<sup>-1</sup>. These isolates were moderately carbendazim resistant (mbc<sup>mR</sup>) and carbendazim-sensitive (mbc<sup>S</sup>), respectively (Figure 3a). Concentrations of 0.1 and 100 carbendazim mg L<sup>-1</sup> were adopted as discriminatory lower inhibitory doses for determining three levels of sensitivity response, in *S. pyricola*. In most isolates a low sensitivity response was independent of the fungicide spray in the field during the year of isolate sampling.

On the spore inoculation tests, individual spores of the isolates ISF-1, E-1 and K-5b germinated on carbendazim concentrations of up to 100 mg L<sup>-1</sup> without differing from the untreated control. However, after three weeks of incubation, only isolates ISF-1 and E-1 grown with 100 mg L<sup>-1</sup>

of the fungicide formed colonies with pycnidia. Isolate K-5b formed very few and restricted colonies with 10 mg L<sup>-1</sup> of carbendazim, while it failed to form any colonies with a carbendazim concentration of 100 mg L<sup>-1</sup>. Isolates ISF-1 and E-1 were therefore characterized as mbc<sup>R</sup> and isolate K-5b as mbc<sup>mR</sup>. With a carbendazim concentration of 0.1 mg L<sup>-1</sup>, spores of the reference isolate M-1 (derived from an unsprayed and isolated garden pear tree) and its 37M-1 mutant germinated with short germ tubes that did not produce any visible colonies. They were therefore designated mbc<sup>S</sup>. The ED<sub>50</sub> values permitting the formation of visible colonies of mbc<sup>R</sup>, mbc<sup>mR</sup> and mbc<sup>S</sup> isolates were >100; 4.115±0.311; and 0.038 to 0.040±0.002 mg L<sup>-1</sup> respectively (Table 2).

#### Sensitivity of strains to the DMI fungicides

The sensitivity response of *S. pyricola* isolates to the three DMI fungicides varied widely, with the MIC values determined by point inoculation ranging from 0.001 to 1 mg L<sup>-1</sup>. Flusilazole was the most effective; it had an MIC lower than 0.005 mg L<sup>-1</sup> with most isolates (80%). The MIC of bitertanol

Table 2. ED<sub>50</sub> values<sup>a</sup> of representative isolates of *Septoria pyricola* to carbendazim (C), bitertanol (B), flusilazole (F), myclobutanil (M), azoxystrobin (A), kresoxim-methyl (K), pyraclostrobin (P), trifloxystrobin (T) and boscalid (Bo).

Isolate	ED <sub>50</sub> values (mg L <sup>-1</sup> ) <sup>a</sup>								
	C	B	F	M	A	K	P	T	Bo
ISF-1	>100	0.017 (0.004) <sup>b</sup>	0.011 (0.0045)	0.021 (0.004)	0.001 (0.00009)	0.0005 (0.00004)	0.0005 (0.00006)	0.001 (0.0001)	0.039 (0.01)
K-5b	4.115 (0.311)	0.0005 (0.00004)	<0.0001 (-)	0.017 (0.002)	0.0002 (0.00002)	0.0004 (0.00004)	<0.0001 (-)	0.001 (0.0002)	0.019 (0.002)
M-1	0.038 (0.002)	0.002 (0.0001)	<0.0001 (-)	0.001 (0.0005)	0.002 (0.0004)	0.017 (0.0008)	0.004 (0.0002)	0.003 (0.0002)	0.014 (0.003)
E-1	>100	0.015 (0.004)	0.009 (0.0045)	0.020 (0.003)	0.0002 (0.00002)	0.002 (0.00009)	0.00014 (0.00002)	<0.0001 (-)	0.035 (0.01)
37M-1	0.040 (0.002)	0.002 (0.0001)	<0.0001 (-)	0.002 (0.0005)	0.027 (0.002)	0.011 (0.0004)	0.005 (0.0002)	0.002 (0.0001)	0.016 (0.003)

<sup>a</sup> The 50% effective dose values are based on inhibition of 50% of visible colonies formed on PDA amended medium with different concentrations of the fungicides for 8 days after inoculation with a spore suspension (approx. 3,000 per mL).

<sup>b</sup> Numbers in parentheses are the standard errors (SE) of the mean ED<sub>50</sub> values over three replicates at *P*=0.05.

had two peaks, at 0.001 (31%) and 0.1 (30%) mg L<sup>-1</sup>. Myclobutanil was the least effective, with a MIC of 0.1 mg L<sup>-1</sup> for only 69% of the isolates (Figure 3b). A few isolates for which the MIC of DMI fungicides was 1 mg L<sup>-1</sup> were detected in pear orchards where these fungicides had been sprayed in the year of sampling; these DMI-resistant isolates were also highly resistant to carbendazim. The mean MICs of bitertanol, flusilazole and myclobutanil were 0.133±0.036, 0.075±0.044 and 0.230±0.038 mg L<sup>-1</sup> respectively. There was a tendency for a cross sensitivity response of the isolates to the three DMIs.

On media amended with 0.0001 mg L<sup>-1</sup> of one of each DMI fungicide and inoculated with the spore suspension, spore germination and germ tube length did not differ from the controls. But as the fungicide concentration was increased, the spore tube length and the colony size gradually decreased. With isolates K-5b, M-1 and 37M-1, a concentration of 0.01 mg L<sup>-1</sup> bitertanol completely inhibited the formation of visible colonies. Myclobutanil at 0.01 mg L<sup>-1</sup> and flusilazole at 0.001 mg L<sup>-1</sup> only enabled the growth of mycelial colonies with short hyphae hardly visible to the naked eye, and these colonies remained sterile, with a diameter of less than 0.3 mm three weeks after inoculation. The less sensitive isolates ISF-1 and E-1 developed mature colonies with a concentration of 0.01 mg L<sup>-1</sup> of bitertanol or myclobutanil, and with 0.001 mg L<sup>-1</sup> of flusilazole, but not with higher fungicide concentrations. The ED<sub>50</sub> values of bitertanol, flusilazole and myclobutanil for visible colony formation of five representative isolates ranged from 0.0005±0.00004 to 0.017±0.004, from <0.0001 to 0.011±0.0045; and from 0.001±0.0005 to 0.021±0.004 mg L<sup>-1</sup> respectively (Table 2).

#### Sensitivity of strains to QoI fungicides

The MICs of the QoIs varied widely, from 0.005 to 10 mg L<sup>-1</sup>. With most isolates, the MICs of the QoIs were up to 0.01 mg L<sup>-1</sup>, except for kresoxim-methyl. Kresoxim-methyl had a 10-fold lower MIC with 72% of the isolates (Figure 3c). Overall, the mean MICs of point-inoculated azoxystrobin, kresoxim-methyl, pyraclostrobin and trifloxystrobin enabling colony formation were 0.177±0.040; 0.075±0.035; 0.067±0.063; and 0.073±0.065 mg L<sup>-1</sup> respectively. One isolate, M-1, collected from an unsprayed garden tree in 2004, showed a 10-fold lower sensitivity to azoxystrobin and trifloxys-

trobin (MIC 1 mg L<sup>-1</sup>). Isolate E-1, obtained in the spring of 2007, with five other isolates, from an orchard with heavy disease incidence and where trifloxystrobin had been sprayed (Table 1), was even less sensitive to azoxystrobin (MIC 10 mg L<sup>-1</sup>). The MICs of the other three QoIs (trifloxystrobin, kresoxim-methyl, pyraclostrobin) with isolate E-1 were 1, 0.1, and 0.1 mg L<sup>-1</sup> respectively. However, after repeated point inoculation testing, with sub-culturing and long storage (about a year) at 5°C, isolate E-1 showed a somewhat greater sensitivity to azoxystrobin (data not shown).

With all the five isolates evaluated by spore inoculation, the percentage of germinated spores and the length of the germ tubes tended to decrease with increasing fungicide concentration. However, at concentrations of 1 mg L<sup>-1</sup> of kresoxim-methyl or trifloxystrobin, only spores of field isolate M-1 (sensitive to a primary MIC of 1 mg L<sup>-1</sup> azoxystrobin) and its laboratory mutant 37M-1 (selected on a medium containing 1 mg L<sup>-1</sup> trifloxystrobin), germinated at a high percentage, giving rise to sparse, whitish, sterile mycelial colonies of up to 0.4 mm in diameter. Similar sterile colonies were formed by these two isolates even at a concentration of 10 mg L<sup>-1</sup> of kresoxim-methyl (data not shown). Isolate E-1, which was initially less sensitive to azoxystrobin (primary MIC 10 mg L<sup>-1</sup>) failed to grow when QoI concentrations exceeded 0.1 mg L<sup>-1</sup>. The ED<sub>50</sub> for visible colony formation of the five representative isolates with azoxystrobin, kresoxim-methyl, pyraclostrobin and trifloxystrobin ranged respectively from 0.0002±0.00002 to 0.027±0.002; from 0.0004±0.00004 to 0.017±0.0008, from <0.0001 to 0.005±0.0002; and from <0.0001 to 0.003±0.0002 mg L<sup>-1</sup> (Table 2).

#### Sensitivity of strains to boscalid

The MICs of boscalid with the isolates ranged from 0.005 to 1 mg L<sup>-1</sup> (Figure 3d) and the mean MIC was 0.111±0.044 mg L<sup>-1</sup>, when the isolates were tested for colony formation by point inoculation. A concentration of 1 mg L<sup>-1</sup> inhibited the formation of colonies with fruiting bodies and was defined as the MIC threshold when screening for sensitivity to boscalid in *S. pyricola*. With dispersed spore inoculations, a concentration of 1 mg L<sup>-1</sup> boscalid failed to inhibit spore germination completely, but it did permit growth of sparse, sterile mycelial colonies, invisible to the naked eye. The



ED<sub>50</sub> values for visible colony formation of the five representative isolates ranged from 0.014±0.003 to 0.039±0.01 mg L<sup>-1</sup> boscalid (Table 2).

## Discussion

Having a simple and reliable method for assessing the sensitivity of plant pathogenic fungi to synthetic fungicides is of great practical use. Adequate information about *S. pyricola*, only a minor pathogen in commercial terms, but of great local importance in Mediterranean pear-producing countries, has hitherto been lacking. The pycnidiospores of *S. pyricola*, unlike those of many filamentous fungi, germinated gradually on PDA, giving rise around the inoculation point to small, compact mycelial colonies consisting of short hyphae with pycnidia. Thus common methods to test fungicide efficacy *in vitro* by measuring either the expansion of the mycelial colony or the number of germinated spores, were found to be inappropriate. It was nevertheless possible to assess fungicide efficacy by recording the visible colonies formed on an amended medium, after either point inoculation or dispersed inoculation of a spore suspension. The reference sensitivity of selected isolates to representative fungicides of the widely used classes of the benzimidazoles and DMIs, and of the more recently introduced QoIs, are shown. A baseline sensitivity of *S. pyricola* populations to the new fungicide boscalid (SDHI) was also determined, before its introduction to agricultural practice.

Both the benzimidazoles and the DMIs inhibit mycelial growth mainly by interfering with either mitotic division (Davidse and Ishii, 1995) or ergosterol biosynthesis (Sisler and Ragsdale, 1983), but they do not adversely affect spore germination. In the present work, *S. pyricola* was found to have a similar response to both classes of fungicides. The assessment of colony formation at various fungicide concentrations indicated that carbendazim-resistant isolates were common. Of 36 randomly selected single-spore isolates, 28 were highly resistant, three had intermediate resistance, and only five were sensitive. There was no correlation between sensitivity to carbendazim, the year of sampling, or the concurrent application of a benzimidazole fungicide in the field. The wide and predominant distribution of resistant phenotypes in

*S. pyricola* populations can be related to the extensive use of benzimidazole fungicides to control pear scab over the last 40 years. As has been found with other pathogens, the benzimidazole-resistant *S. pyricola* strains have good environmental fitness and can survive and compete with the wild strains even after a long cessation of fungicide pressure (Dovas *et al.*, 1976; Pappas, 1997). This is consistent with the generally inadequate level of *S. pyricola* control provided by the benzimidazole-based fungicides in Greece. As has been reported for pear orchards naturally infected with *S. pyricola*, spraying with either benomyl (Biris *et al.*, 1991), or carbendazim (Pappas *et al.*, 2006), does not effectively control leaf fleck. In addition, carbendazim was completely ineffective in controlling leaf fleck when pear trees were mechanically inoculated with a mixture of a carbendazim-sensitive and a carbendazim-resistant strain (Chatzidimopoulos and Pappas, 2008).

As has been shown in early studies, the effectiveness of the DMI fungicides involves many genes (Van Tuyl, 1977). For this reason, a gradual but low-level and rather reversible resistance is arising in the field due to lack of fitness (Brent and Hollomon, 2007). Our results show a wide range of sensitivity to bitertanol, flusilazole and myclobutanil, suggesting a creeping reduction in the sensitivity response of *S. pyricola*, due to continuous selection pressure provided by DMI fungicides sprayed over a long period. With most of the *S. pyricola* isolates, colonies were inhibited by the DMIs at a MIC of up to 0.1 mg L<sup>-1</sup>. A few isolates exhibited cross-insensitivity to three of the DMIs and required 10-fold higher MICs. However, none of the isolates formed mycelial colonies with pycnidia when the concentration of bitertanol or myclobutanil in the medium exceeded 0.01 mg L<sup>-1</sup> or when the concentration of flusilazole exceeded 0.001 mg L<sup>-1</sup>. Flusilazole was the most effective fungicide against *S. pyricola*, and myclobutanil was the least effective. Such differences in the intrinsic effectiveness of the DMI fungicides were consistent with previous reports on other pathogens (Ypema *et al.*, 1997; Wong and Midland, 2007). The isolates less sensitive to the DMIs in the tests were also resistant to carbendazim, presumably because of the long period of extensive benzimidazole application in the field. The occurrence of *S. pyricola* isolates with a

10-fold lower sensitivity to bitertanol, flusilazole and myclobutanil, although it is not frequent, highlights the risk of a decline in pear leaf speck control with these DMI fungicides and needs further investigation. In the case of *S. tritici* on cereals (Turner *et al.*, 1996), of *Cercospora beticola* on sugar beet (Karaoglanidis *et al.*, 2002), and of other plant pathogens (Köller, 1991), a 2- to 5-fold reduction in sensitivity has been associated with a history of intensive DMI spraying over several years, and has been correlated with a lower fungicide effectiveness in the field.

The activity of quinone outside inhibitors was related to the fact that this class of fungicides binds to the cytochrome *bc1* (complex III) at the Qo site (Bartlett *et al.*, 2002) and the effectiveness of boscalid was due to its inhibiting succinate-ubiquinone reductase activity in complex II (BASF Corporation, 2003). Both groups of compounds block the electron transport chain in sensitive fungi, but with different target positions. Because of their mode of action, these fungicides are prone to produce resistance in the pathogens they are intended to control. *Alternaria alternata* strains resistant to boscalid (Avenot and Michailides, 2007) and more generally various plant pathogen strains resistant to the QoIs (Heaney *et al.*, 2000; Bartlett *et al.*, 2002; Fungicide Resistance Action Committee, 2009), have already been reported. In wild fungal populations there seems to be a broad range of sensitivity response to the novel fungicide boscalid (SDHI) and to members of the recently introduced QoI class (Myresiotis *et al.*, 2008; Wise *et al.*, 2008). In the present work on *S. pyricola*, the MICs of both these groups of fungicides ranged mostly from 0.005 to 0.1 mg L<sup>-1</sup>, as shown by colony formation on a medium inoculated with a spore suspension. Only one out of 36 isolates was less sensitive to boscalid, but even this isolate failed to produce colonies with pycnidia at a boscalid concentration of 1 mg L<sup>-1</sup>. On the whole the QoIs were intrinsically more effective than boscalid.

It was interesting to note that two *S. pyricola* isolates, M-1, from an unsprayed garden tree, and E-1, from a trifloxystrobin-treated orchard, were, when initially tested, up to 10- and 100-fold less sensitive to azoxystrobin respectively. These isolates were also less sensitive to the other QoI fungicides, though not as much as they were to

azoxystrobin. In accordance with earlier reports on other fungal species, isolate E-1 tended to become more sensitive after long maintenance and repeated sub-culturing on a fungicide-free medium. Miguez *et al.* (2003) reported that the growth of *S. tritici* strains resistant to azoxystrobin led to alternative oxidase activation (AOX). Some *S. tritici* isolates became less resistant when they were subcultured. However, none of these isolates had the G143A mutation in the cytochrome *b* gene, which confers stable resistance on the QoIs. It is known that AOX provides a rescue from QoI action in various fungal species (Avila-Adame and Köller, 2003; Miguez *et al.*, 2003; Wood and Holomon, 2003). Such a mechanism also seems to occur in *S. pyricola* since kresoxim-methyl activity increased 30-fold when 5 mg L<sup>-1</sup> salicylhydroxamic acid (SHAM), an AOX inhibitor, was present (Mylonopoulos, personal communication). When QoI selection pressure is long absent, however, the AOX function is probably eliminated, leading to isolates eventually becoming more sensitive to the QoI fungicides. In asexual reproduction, the unstable response to QoIs reflects the status of the mitochondrial heteroplasm, in which the wild-type DNA dominates the mutated DNA, leading to a greater sensitivity to the fungicide (Ishii *et al.*, 2007). Even so, the cytochrome *b* mutation is in the mitochondrial genome, so that the inheritance of resistance to QoIs is presumably non-Mendelian (Gisi *et al.*, 2002).

In conclusion, this work describes a method of spore inoculation to assess the effectiveness of fungicides against *S. pyricola*, and demonstrates the sensitivity of the reference isolates to benzimidazole compounds, and to the DMI, QoI and SDHI fungicides. It documents for the first time the high frequency of *S. pyricola* isolates with resistance to carbendazim and the occurrence of some isolates with a 10-fold lower sensitivity response to the DMIs bitertanol, flusilazole and myclobutanil. The implications of these findings for the control of leaf fleck need further investigation. With some exceptions, the recently introduced fungicides of the QoI class, and boscalid (SDHI), were very effective *in vitro* against most *S. pyricola* isolates and offered a promising alternative for leaf fleck control in pear. However, since there is a high risk that *S. pyricola* will develop resistance to these target-specific fungicides, they should be applied only preventively and with caution.

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