

SENSITIVITY OF *Venturia inaequalis* CHILEAN ISOLATES TO DIFENOCONAZOLE, FENARIMOL, MANCOZEB, AND PYRIMETHANIL

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ABSTRACT

Apple scab (*Venturia inaequalis*) is the most important disease in Chilean apple (*Malus domestica* Borkh.) orchards where fungicide management is the main tool to control disease. Sensitivity tests to difenoconazole, fenarimol, mancozeb, and pyrimethanil fungicides were conducted on *V. inaequalis* wild isolates to construct local baseline sensitivity distributions, and compare them with a collection of isolates from seven different commercial orchards with no evidence of practical resistance. *In vitro* assays were conducted and sensitivity was measured as inhibition of mycelial growth (difenoconazole, fenarimol, pyrimethanil) or conidial germination (mancozeb). Departure from the baseline distribution to less sensitivity was found for difenoconazole, fenarimol, and mancozeb with resistance factors of 4.7, 5.8, and 2.1, respectively. The highest difference between baseline and orchard populations was observed for fenarimol which showed the highest shift to less sensitivity. Discriminatory doses of 0.04, 1.0, 0.6, and 0.2 $\mu\text{g mL}^{-1}$ are proposed for *in vitro* monitoring of sensitivity to difenoconazole, fenarimol, mancozeb, and pyrimethanil, respectively.

Key words: Apple, apple scab, fungicides, DMI fungicides, dithiocarbamates, anilinopyrimidines.

INTRODUCTION

Apple scab incited by *Venturia inaequalis* (Cooke) Winter (anamorph *Spilocaea pomi* Fr.) is the most important disease in apples grown in Chile. Chemical control with fungicides has been the main tool to control disease and fungicides are sprayed when there are favorable conditions for disease development or at fixed intervals depending on the growing season, area of production, and climatic conditions. Failure to control disease has been observed in some orchards and fungicide resistance is claimed to be the main reason, although there is only one published study about fungicide resistance (Sallato and Latorre, 2006) reporting on resistance to strobilurin kresoxym methyl in southern Chile. There are some unpublished studies from the chemical industry testing the sensitivity of the pathogen's local isolates to commonly used fungicides. Data obtained are normally compared with standards found in the literature with data gathered from studies conducted elsewhere. There are no studies about the sensitivity of wild local isolates, and there is a

lack of information on baseline sensitivities to some old and commonly used fungicides.

Fungicide resistance in *V. inaequalis* is well documented worldwide for old fungicides such as dodine (Köller *et al.*, 1999; Broniarek-Niemiec and Bielenin, 2008), benzimidazole (Koenraadt *et al.*, 1992), demethylation inhibitors (DMIs) (Sholberg and Haag, 1993; Köller *et al.*, 1997), and newer fungicides such as strobilurins (Olaya and Koller, 1999; Fontaine *et al.*, 2009) and anilinopyrimidines (Küng *et al.*, 1999). There are no reports in the literature about sensitivity to mancozeb, an old fungicide still used for apple scab control, used alone or mixed mainly with DMIs as an anti-resistance strategy. The objectives of this research were to determine the sensitivity of wild Chilean populations to the difenoconazole, fenarimol, mancozeb, and pyrimethanil fungicides, and to compare them with the sensitivity of isolates obtained from commercial orchards under conventional fungicide management.

MATERIALS AND METHODS

Fungal isolates

Venturia inaequalis wild isolates were obtained from apple trees located far away from commercial orchards

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having no history of fungicide treatments from central to southern Chile in 2007 and 2008. Isolates from seven commercial orchards, with a long history of fungicide treatments, were collected in central and southern Chile in 2008 and 2009. These orchards had low levels of apple scab and failure to control disease was not observed.

Monoconidial cultures

Individual scab lesions on apple leaves were washed with 100 μL sterile distilled water (SDW), put in water agar medium, and incubated at 20 ± 1 °C. After 18 to 24 h, single germinating *V. inaequalis* conidia were transferred to acidified potato dextrose agar (aPDA) plates (0.5 mL lactic acid per liter of medium). A culture was collected and monoconidial isolates replicated to obtain enough fungal material to conduct sensitivity tests. A total of 50 wild isolates and 50 orchard isolates were tested for each fungicide since a higher number does not increase test precision (Smith *et al.*, 1991).

Fungicides

Commercial formulations were employed for sensitivity assays; difenoconazole (Score 250 EC, Syngenta, Basel, Switzerland), fenarimol (Rubigan 12 EC, Gowan, Yuma, Arizona, USA), mancozeb (Dithane M 45 WP, Dow Agro Science, Indianapolis, Indiana, USA), and pyrimethanil (Scala 400 SC, Bayer Crop Science, Leverkusen, Germany).

Sensitivity assays

Venturia inaequalis sensitivity to mancozeb was determined according to a previously described procedure (Köller *et al.*, 1999) in brief conidial suspensions in SDW obtained from 4 to 6 wk old monoconidial cultures where 30 to 50 μL were spread on aPDA amended with the tested fungicide in concentrations ranging from 0 to 10 $\mu\text{g mL}^{-1}$ and incubated for 24 h. Germination of 100 conidia was assessed in four replicate plates for each fungicide concentration. A conidium was considered germinated when its germinating tube was at least 1.5 times its own length.

Venturia inaequalis to difenoconazole and fenarimol, two DMI fungicides, was determined for mycelial growth on aPDA amended with the respective fungicide in concentrations ranging from 0 to 10 $\mu\text{g mL}^{-1}$ in four replicate plates. A small piece of mycelium, about 1 mm in diameter, was transferred from monoconidial cultures to fungicide amended plates and four different isolates were placed in a Petri dish. The mean colony diameter was measured after 4 wk of incubation at 20 ± 1 °C. Sensitivity to pyrimethanil was determined for the inhibition of mycelial growth in a synthetic medium consisting of 10 g glucose; 1.5 g K_2HPO_4 , 2 g KH_2PO_4 , 1 g $(\text{NH}_4)_2\text{SO}_4$,

0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 12.5 g agar per liter of medium (SDW) (Leroux *et al.*, 1999).

The effective concentration to inhibit mycelial growth or conidial germination by 50% (ED_{50}) was estimated by performing a regression between the percentage of mycelial growth inhibition or conidial germination against the fungicide concentration logarithm. Values of the ED_{50} frequency distribution were used to construct population sensitivity curves. ED_{50} values from wild and orchard isolates were log transformed and analyzed by the Kolmogorov and Smirnov normality test (Lilliefors, 1967). Mean wild and orchard population sensitivities were subjected to ANOVA (Minitab, State College, Pennsylvania, USA). Discriminatory doses to separate sensitive from resistant isolates were proposed based on wild sensitivities according to Köller *et al.* (1991). Resistance factors (RF) were calculated for each fungicide as the ratio between mean sensitivity of the orchard population divided by the mean sensitivity of the wild population (Kunz *et al.*, 1997).

RESULTS AND DISCUSSION

Distribution of sensitivities of wild and orchard populations were single lognormal according to the Kolmogorov and Smirnov test for difenoconazole ($p = 0.12$ and $p > 0.15$); fenarimol ($p = 0.05$ and $p > 0.10$); mancozeb ($p > 0.15$); and pyrimethanil ($p = 0.08$ and $p > 0.15$), respectively.

Baseline sensitivity to difenoconazole of the wild population ranged from 0.00001 to 2.9 $\mu\text{g mL}^{-1}$ resulting in a separation factor of 290 000 (ratio between the least and most sensitive isolate of the population) (Table 1). A tenfold amplitude decrease in the separation factor was observed for the orchard population which fluctuated from 0.0002 to 5.8 $\mu\text{g mL}^{-1}$ (Table 2), reflecting the loss of the most sensitive isolates and the presence of new and less sensitive isolates absent in the wild population. Accordingly, the sensitivity distribution curve of the orchard population has been displaced to the right (Figure 1), reaching a mean sensitivity of 0.08 $\mu\text{g mL}^{-1}$ significantly different from the baseline sensitivity of 0.017 $\mu\text{g mL}^{-1}$ ($p = 0.002$). The wild isolates showed a high sensitivity to difenoconazole and reflected a great intrinsic activity of this fungicide when compared to fenarimol's lower activity. Similar results were obtained by Kunz *et al.* (1997) when assessing the sensitivity of *in vivo* DMIs where difenoconazole was ranked as the most active fungicide against *V. inaequalis* when compared to flusilazol, fenarimol, tebuconazole, and pyrifenofox.

Difenoconazole has been used for more than a decade to control apple scab in Chile and it is the latest DMI fungicide introduced to control it. It is regarded as one

Table 1. Sensitivity distribution (ED₅₀) of wild and orchard populations of *Venturia inaequalis* to tested fungicides.

Fungicides	Range ED ₅₀	Mean ED ₅₀	DD
		$\mu\text{g mL}^{-1}$	
Wild population			
Difenoconazole	0.00001 - 2.9	0.017	0.04
Fenarimol	0.0003 - 1.4	0.09	1.0
Mancozeb	0.046 - 6.5	0.44	0.6
Pyrimethanil	0.0037 - 2.3	0.10	0.2
Commercial orchard population			
			Resistance Factor
Difenoconazole	0.0002 - 5.8	0.08	4.7
Fenarimol	0.0048 - 5.1	0.53	5.8
Mancozeb	0.0046 - 20.0	0.93	2.1
Pyrimethanil	0.0061 - 3.2	0.14	1.4

ED₅₀: The effective concentration to inhibit mycelial growth or conidial germination by 50%.

DD: Discriminatory dose proposed in this study.

Resistance factor: Mean ED₅₀ of the orchard population divided by mean ED₅₀ of wild population.

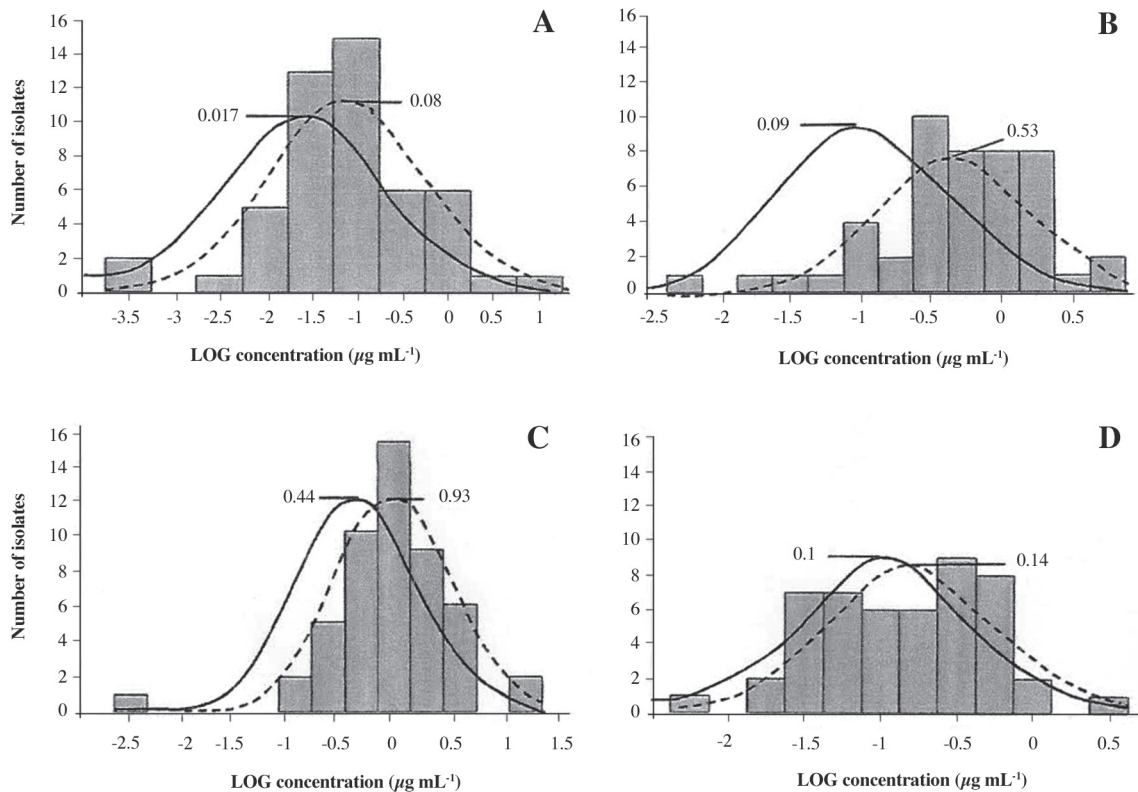
of the most effective fungicides which is widely used alone or mixed with a multisite fungicide as a resistance management strategy (Köller and Wilcox, 1999; 2000). Our results showed that orchard populations have lost sensitivity to difenoconazole, but there is still a proportion of sensitive isolates. This is considered to be a good situation compared with narrow sensitivity distributions where fungicide resistance can be acquired faster (Köller *et al.*, 1991). Nevertheless, an RF value of 4.7 was calculated for this fungicide, thus indicating a possible decrease in field activity. Smith *et al.* (1991) reported loss of field activity of DMI fungicides with RF values over 3. According to baseline sensitivity, a concentration of 0.04 $\mu\text{g mL}^{-1}$ is proposed as a discriminatory dose for *in vitro* monitoring of sensitivity to difenoconazole.

Sensitivity of the wild population to fenarimol ranged from 0.0003 to 1.4 $\mu\text{g mL}^{-1}$ with a separation factor of 4667 and a mean sensitivity value of 0.09 $\mu\text{g mL}^{-1}$ (Table 1). The sensitivity of the orchard population was narrowed down to a separation factor of 1062, thus reflecting the loss of the most sensitive part of the population and an increase of less sensitive isolates. A great departure from the baseline population was observed for fenarimol when compared to difenoconazole (Figure 1), and where a larger part of the population was to the right. Accordingly, a higher resistance factor (RF = 5.8) was calculated. Mean sensitivity values of the wild and orchard populations were significantly different ($p = 0.001$). The wild population showed a wide spectrum of sensitivities to fenarimol as expressed by the separation factor of 4667, which is greater than the value of 60 previously reported for this fungicide (Köller *et al.*, 1991).

This data reflects important differences in fenarimol

sensitivity of Chilean isolates and populations from elsewhere. Fiaccadori *et al.* (1987) reported a mean ED₅₀ of 0.04 $\mu\text{g mL}^{-1}$ for fenarimol in unexposed isolates from Italy and Holland, whereas the same value was obtained by Köller *et al.* (1991) for New York isolates, while Thind *et al.* (1986) obtained a mean ED₅₀ of 0.03 $\mu\text{g mL}^{-1}$. Accordingly, our results of a discriminatory dose of 1.0 $\mu\text{g mL}^{-1}$ is suggested for the *in vitro* determination of sensitivity to fenarimol, which is higher than the 0.05 $\mu\text{g mL}^{-1}$ reported by Köller *et al.* (1991) and Smith *et al.* (1991). Therefore, the use of discriminatory doses obtained in foreign studies for testing *in vitro* sensitivity of Chilean isolates to fenarimol would be misleading.

Baseline sensitivity to mancozeb varied from 0.046 to 6.5 $\mu\text{g mL}^{-1}$ (Table 1) representing a separation factor of 141, while the orchard population had a separation factor greater than 2000 with sensitivity values varying from 0.0046 to 20 $\mu\text{g mL}^{-1}$ (Table 2). A wider distribution of sensitivities observed in the orchard population for this fungicide resulted from the occurrence of few highly sensitive isolates not present in the baseline population studied, as well as the increase of new and less sensitive isolates (Figure 1). The mean sensitivity of the wild (0.44 $\mu\text{g mL}^{-1}$) and orchard (0.93 $\mu\text{g mL}^{-1}$) ($p = 0.001$) populations were significantly different and RF was calculated as 2.1. Orchard isolates, 2 to 4 times less sensitive to the fungicide dodine, were related to failure to control apple scab (McHardy, 1996). Both dodine and mancozeb are multisite fungicides and similar shifting of sensitivity from baseline populations could be expected in orchard populations. Mancozeb is an old fungicide widely used to control apple scab and still mainly employed mixed



ED₅₀: The effective concentration to inhibit mycelial growth or conidial germination by 50%.

Figure 1. Sensitivity frequency distribution of *Venturia inaequalis* orchard isolates to tested fungicides and mean ED₅₀ (discontinuous line) compared to baseline sensitivity and mean ED₅₀ (continuous line). A. Difenconazole; B. Fenarimol; C. Mancozeb; D. Pyrimethanil.

with more specific fungicides as an anti-resistance strategy. Very little difference was observed among baseline and orchard populations, which could be the result of a lower resistance pressure or the intrinsic lesser likelihood of building up resistance due to its multisite mode of action. A concentration of $0.6 \mu\text{g mL}^{-1}$ is proposed as a discriminatory dose for *in vitro* determination of sensitivity to mancozeb.

Sensitivity of the baseline population to pyrimethanil was narrow and fluctuated from 0.0037 to $2.3 \mu\text{g mL}^{-1}$ with a separation factor of 621 (Table 1). Narrower

distributions were described by Köller *et al.* (2005) in a sensitivity study of 22 isolates from New York which could result from the small number of tested isolates. A very similar distribution of sensitivities was observed in this study for the orchard population which ranged from 0.0061 to $3.2 \mu\text{g mL}^{-1}$ with a separation factor of 524 (Table 2, Figure 1). The differences in mean sensitivity values of wild and orchard populations which were not significant ($p = 0.26$) and an RF value of 1.4 indicate that sensitivity of *V. inaequalis* orchard isolates to pyrimethanil has not

Table 2. Distribution of sensitive ED₅₀ ($\mu\text{g mL}^{-1}$) of orchard population of *Venturia inaequalis* to the fungicides tested.

	ED ₅₀ Range	Mean ED ₅₀	Resistance factor ¹
Difenconazole	0.0002-5.8	0.08	4.7
Fenarimol	0.00048-5.1	0.53	5.8
Mancozeb	0.0046-20.0	0.93	2.1
Pyrimethanil	0.0061-3.2	0.14	1.4

¹Mean ED₅₀ of the orchard population divided by the mean ED₅₀ of the wild type population.

ED₅₀: The effective concentration to inhibit mycelia growth or conidial germination by 50%.

significantly moved from the baseline. A discriminatory dose of $0.2 \mu\text{g mL}^{-1}$ is suggested for *in vitro* determination of sensitivity to pyrimethanil. The same dose was determined by Köller *et al.* (2005) for the New York isolates.

CONCLUSIONS

A departure from the baseline population of *V. inaequalis* isolates from commercial orchards was demonstrated for the first time for difenoconazole, fenarimol, and mancozeb in Chile. The departure from baseline sensitivity for fenarimol was considerably higher than for difenoconazol. Fenarimol exhibited the highest RF among the tested fungicides. The presence of isolates highly sensitive to fungicides tested in orchard populations indicates a slow process of sensitivity loss, and if adequate resistance management is undertaken, resistance to the studied fungicides will be delayed. It is important to note that the loss of sensitivity to pyrimethanil of orchard populations was not found, thus subsequent monitoring and appropriate anti-resistance management would delay acquisition of resistance. The results presented are representative of a region, not of particular orchards with failure to control apple scab, and specific situations should be analyzed in detail to better understand the local response of *V. inaequalis* isolates to fungicides used to control it.

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RESUMEN

Sensibilidad de aislados chilenos de *Venturia inaequalis* a difenoconazole, fenarimol, mancozeb y pyrimethanil. La sarna del manzano (*Venturia inaequalis*) es la principal enfermedad del cultivo en Chile y es controlada principalmente con el uso de fungicidas. Se realizaron pruebas de sensibilidad a los fungicidas difenoconazole, fenarimol, mancozeb y pyrimethanil en aislados monoconidiales de cepas silvestres de *V. inaequalis* con el fin de elaborar curvas basales de sensibilidad y compararlas con la sensibilidad de una colección de aislados monoconidiales provenientes de siete huertos comerciales sin evidencias de resistencia práctica. Se realizaron pruebas *in vitro* donde la sensibilidad se midió como la inhibición del crecimiento miceliar (difenoconazole, fenarimol y pyrimethanil) o la inhibición de la germinación de conidias (mancozeb). La población de huertos comerciales estudiada presentó una menor sensibilidad a los fungicidas difenoconazole,

fenarimol y mancozeb con valores de factor de resistencia 4,7; 5,8; y 2,1 para difenoconazole, fenarimol y mancozeb, respectivamente. La mayor diferencia de sensibilidad entre la población silvestre y la de huerto se obtuvo con fenarimol, fungicida para el cual se observó el mayor cambio hacia una menor sensibilidad. Se proponen dosis discriminatorias de 0,04; 1,0; 0,6; y $0,2 \mu\text{g mL}^{-1}$ para difenoconazole, fenarimol, mancozeb y pyrimethanil, respectivamente.

Palabras clave: manzano, sarna del manzano, fungicidas, fungicidas IBE, ditiocarbamatos, anilino pirimidinas.

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