Sensitivity of *Venturia inaequalis* to myclobutanil, penconazole and dodine in relation to fungicide use in Hawke's Bay apple orchards

R.M. Beresford¹, P.J. Wright², P.N. Wood³ and N.M. Park³

¹The New Zealand Institute for Plant & Food Research Ltd, Mt Albert Research Centre, Private Bag 92169, Auckland 1142, New Zealand

²The New Zealand Institute for Plant & Food Research Limited, Cronin Road, RD1 Pukekohe, New Zealand

³The New Zealand Institute for Plant & Food Research Ltd, Hawke's Bay Research Centre, Private Bag 1401, Havelock North, Hastings 4157, New Zealand

Corresponding author: robert.beresford@plantandfood.co.nz

Abstract A survey of Integrated Fruit Production and organic apple orchards in Hawke's Bay in 2010-11 investigated sensitivity of *Venturia inaequalis* isolates to demethylation inhibitor (DMI) and dodine fungicides. EC₅₀ values from mycelial growth assays for two DMIs showed continued loss of sensitivity since the 1990s. Sensitivity was lower in orchards where DMI usage exceeded resistance management guidelines. The most DMI-sensitive isolates were found in one organic orchard. Plant inoculations showed that black spot control of myclobutanil-resistant *V. inaequalis* strains by one application of myclobutanil was only 55%, compared with 99% for myclobutanil-sensitive strains. The study suggests that resistance to DMI fungicides has continued to develop, that loss of black spot control could be expected in some orchards and that DMI resistance management guidelines need reviewing. There was high sensitivity to dodine in all orchards (mean EC₅₀ of 0.24 mg/litre) and no evidence of increased resistance since the 1990s.

Keywords fungicide resistance, apple scab, demethylation inhibitor, dodine, resistance management.

INTRODUCTION

New Zealand apple industry spray programmes for control of black spot (scab), caused by *Venturia inaequalis*, typically use 16-20 fungicide applications each season (Beresford & Manktelow 1994), including dodine and fungicides in the demethylation inhibitor (DMI) group. These fungicides are particularly important to orchardists because their systemic activity prevents black spot development when they are

applied after infection has occurred. However, these fungicides are also known overseas to select for resistance in *V. inaequalis*.

DMI fungicides were first introduced during the 1970s, and since then many new DMI active ingredients with improved activity have been released. Resistance of *V. inaequalis* to DMIs was first reported in the 1980s in the USA (Stanis & Jones 1985) and has since occurred in Canada,

UK, Europe and Australia. The first New Zealand DMI survey during the 1990-91 growing season (Whelan et al. 1992) reported a national mean concentration that inhibited mycelial growth by 50% (EC_{50}) for myclobutanil of 0.16 mg/litre and 0.039 mg/litre for penconazole. A survey in 1993-94 (Bakker et al. 1995) found a shift towards decreased sensitivity for several DMI active ingredients between 1990-91 and 1993-94. The current New Zealand DMI resistance management guidelines assume that *V. inaequalis* is cross-resistant to all DMI active ingredients. The guidelines recommend a maximum of four DMI applications per season and mixing each application with a fungicide from a different cross-resistance group (Beresford 2005a).

Dodine has been in use worldwide since 1959 and although widespread resistance has reduced its use overseas (Chapman et al. 2011), dodine still appeared to be effective in New Zealand in 1993-94 with a mean EC_{50} of 0.85 mg/litre (Bakker et al. 1995), and in 1997-98 with a mean EC_{50} of 0.527 mg/litre (Bakker 1999). Current New Zealand guidelines are for a maximum of three dodine applications per season (Beresford 2005b).

Because *V. inaequalis* sensitivity to DMI fungicides and dodine has not been investigated since the 1990s, this study sought to update sensitivity information for Hawke's Bay orchards, where 60% of New Zealand's export apple production occurs. It also investigated relationships between fungicide sensitivity, fungicide use and fungicide resistance management guidelines. Of five DMI active ingredients currently registered for use on apples in New Zealand, myclobutanil and penconazole were chosen because they have been widely used in New Zealand since the early 1980s and their sensitivity has previously been quantified (Whelan et al. 1992; Bakker et al. 1995; Bakker 1999).

MATERIALS AND METHODS

Orchard sampling, disease incidence, sensitivity tests and fungicide use

Fifteen Hawke's Bay apple orchards were sampled during December 2010. Orchards with a range of usage of both DMI and dodine fungicides were

selected using 4 years of data from the Pipfruit New Zealand Inc. spray diary database. Included were three organic orchards, which had received no synthetic fungicides for more than 10 years, and 12 Integrated Fruit Production (IFP) orchards (Table 1). Diseased fruit were collected from throughout individual orchard blocks with known fungicide use history. At the same time as sample collection, incidence of black spot was assessed on attached leaves (percentage of leaves infected on 50 shoots) and fruit (percentage of fruit infected in a 200-fruit sample) in each orchard.

Mycelial growth of *V. inaequalis* on fungicide-amended potato dextrose agar (PDA) in Petri plates was tested for the 16-20 isolates successfully obtained from each orchard (Table 1). *Venturia inaequalis* conidia were scraped from sporulating fruit lesions and streaked onto water agar. After 48 h incubation at 20°C, individual germinating conidia were removed and placed onto PDA amended with penicillin (100 mg/litre) and streptomycin sulphate (100 mg/litre) to inhibit bacteria. Colonies were incubated on a laboratory bench at 18-20°C and were sub-cultured, if necessary, to eliminate contaminants. A total of 271 isolates was obtained. Agar discs from the edges of growing colonies were placed on fungicide-amended PDA plates. Each isolate was tested at five concentrations of each fungicide and on unamended control PDA plates. Duplicate plates were used for all tests. Fungicide concentrations were: (1) myclobutanil (Systhane® 400 WP; 400 g/kg myclobutanil) at 0.05, 0.1, 0.2, 0.4 and 0.8 mg/litre, (2) penconazole (Topas® 200 EW; 200 g/litre penconazole) at 0.02, 0.04, 0.08, 0.15 and 0.30 mg/litre and (3) dodine (technical grade dodine) at 0.5, 1.0, 2.0, 4.0 and 8.0 mg/litre.

Diameters of *V. inaequalis* colonies were measured after 42 days incubation at 18-20°C, and mycelial growth was expressed relative to the growth diameter of the unamended control for each isolate. EC_{50} values were calculated from linear regression of logit relative colony diameter relative to $\log_{10}$ fungicide concentration.

Numbers of applications of products containing myclobutanil, penconazole, other DMI fungicides and dodine in the 15 sampled orchards

were summarised for the 2007-2008, 2008-2009, 2009-2010 and 2010-2011 seasons, up to the time the *V. inaequalis* samples were taken.

Plant inoculations with isolates resistant and sensitive to myclobutanil

To investigate the effect of myclobutanil application on black spot development caused by the most DMI-resistant *V. inaequalis* isolates found in the study, 2-year-old potted 'Gala' apple trees were inoculated with a mixture of

four of the most resistant isolates and a mixture of four of the most sensitive isolates (Table 2). Conidia for inoculation were produced from PDA cultures growing on a cellophane layer to promote sporulation. A single spray application was made of three fungicide treatments: (1) water control, (2) myclobutanil (Systhane® 400 WP containing 400 g/litre myclobutanil) applied at 0.12 g/litre and (3) flusilazol, another DMI fungicide (Nustar® containing 200 g/kg flusilazol) applied at 0.15 g/litre).

Table 1 Sensitivity of *Venturia inaequalis* isolates, shown as mean concentration that inhibited mycelial growth by 50% (EC₅₀), to two demethylation inhibitor (DMI) fungicides (myclobutanil and penconazole) and to dodine. Isolates were obtained from two apple cultivars in Hawke's Bay apple orchards. Data for each orchard include the number of fungicide applications per season, calculated from the mean over four seasons from 2007-2008 until the time of sample collection in the 2010-2011 season and black spot incidence (%) in the 2010-2011 season. Bold numbers indicate that the maximum number of DMI or dodine applications recommended in resistance management guidelines was exceeded in at least one season.

Orchard	Cultivar ¹	Production system	Black spot		No. isolates	Mean EC ₅₀ (mg/litre)			No. applications per season (4-year mean)			
			Fruit	Leaves		Mycl.	Penc.	Dodine	Mycl.	Penc.	DMI total	Dodine
1	RG	IFP ²	1.7	1.8	16	0.99	0.20	0.15	0	1.3	1.8	3.0
2	RG	IFP	0.3	2.1	19	0.52	0.07	0.09	0	1.3	1.7	2.8
3	RG	IFP	0.0	1.0	18	0.95	0.19	0.19	0	1.3	1.9	3.0
4	RG	IFP	0.2	3.0	18	0.34	0.08	0.18	0	1.8	2.0	2.8
5	RG	IFP	0.8	1.3	17	0.82	0.15	0.14	0	1	1.5	2.8
6	RG	IFP	1.5	1.0	20	0.79	0.16	0.13	0	2	2.7	2.3
7	RG	IFP	0.5	4.2	19	1.20	0.22	0.34	3.3	0.8	4.3	3.3
8	RG	IFP	0.4	5.2	16	1.12	0.15	0.48	4.3	0.3	4.3	4.9
9	RG	IFP	0.0	0.9	20	0.89	0.17	0.23	3.4	0.3	4.7	3.7
10	RG	IFP	1.7	2.9	18	0.64	0.16	0.54	0	3.0	3.3	2.8
11	RG	IFP	2.7	8.0	20	0.69	0.08	0.39	0.3	2.5	2.3	3.0
12	RG	IFP	0.2	2.1	19	0.39	0.06	0.36	0	2.0	2.3	0.8
13	B	Organic	0.3	1.1	16	0.45	0.06	0.12	0	0	0	0
14	B	Organic	0.0	1.5	18	0.38	0.06	0.17	0	0	0	0
15	B	Organic	1.6	2.6	20	0.11	0.02	0.12	0	0	0	0
Mean EC ₅₀						0.69	0.12	0.24	Max. applications/season		4	3

¹RG= 'Royal Gala'; B= 'Braeburn'.

²IFP= Integrated Fruit Production.

Potted trees with 5-10 leaves per shoot were inoculated with 1×10^5 *V. inaequalis* conidia/ml, comprising equal mixtures of the appropriate isolates. After inoculation, plants were incubated at high relative humidity in a plastic tent for 24 h, and then the fungicide treatments were sprayed to the point of runoff. Six replicate shoots were used for each treatment. After fungicide treatment, the trees were placed in a glasshouse for 14 days then visually assessed for percentage of leaf area with sporulating black spot lesions on the inoculated leaves. When six non-inoculated, non-fungicide-treated trees were checked for background *V. inaequalis* infection, their mean leaf area affected by black spot was 1.3%.

Statistical analysis

Statistical analyses were done using Genstat version 8.1. ANOVA was used to identify significant treatment effects, with mean separation by Fisher's least significant difference (LSD, $P < 0.05$). ANOVA of fungicide sensitivity used $\log_{10}$ EC₅₀ values, rather than EC₅₀ values, to improve normality and variance homogeneity of the data. Disease severity data in the potted plant study did not require transformation.

RESULTS

Myclobutanil sensitivity

Mean EC₅₀ values to myclobutanil for *Venturia inaequalis* isolates from individual orchards showed 11-fold variation, from 0.11 to 1.20 mg/litre (Figure 1). Isolates from the three organic

orchards had relatively low EC₅₀ values and the two highest values occurred in IFP orchards 7 and 8, both of which had exceeded in previous years the numbers of DMI applications recommended for resistance management. Although significant differences in sensitivity were detected between some orchards, overall there was little statistical separation of mean EC₅₀ values. This reflected very high variability in EC₅₀ values among the individual isolates, which ranged from 0.07 to 11.35 mg/litre (Table 2).

Venturia inaequalis isolates in this study were generally less sensitive to myclobutanil than previously reported in New Zealand. The mean EC₅₀ over all orchards of 0.69 mg/litre (Table 1) was higher than the previous national means of 0.16 mg/litre (Whelan et al. 1992) and 0.43 (Bakker et al. 1995). Previous Hawke's Bay EC₅₀ values for myclobutanil were 0.10 mg/litre (Whelan et al. 1992) and 0.48 mg/litre (Bakker et al. 1995).

Penconazole sensitivity

Mean EC₅₀ values to penconazole for isolates from the individual orchards showed 9-fold variation, from 0.02 to 0.22 mg/litre (Figure 1). As was the case for myclobutanil, isolates from the three organic orchards had amongst the lowest EC₅₀ values and isolates from IFP orchards that exceeded resistance management recommendations (orchards 7, 8 and 9) had relatively high EC₅₀ values. The overall mean EC₅₀ value for penconazole of 0.12 mg/litre (Table 1) was considerably higher than the national mean of 0.039 mg/litre reported by Whelan et al.

Table 2 EC₅₀ (mean fungicide concentration that inhibited mycelial growth by 50%) values (mg/litre) for myclobutanil and penconazole for four of the most demethylation inhibitor (DMI)-sensitive and four of the most DMI-resistant *Venturia inaequalis* isolates from this study.

Sensitive isolates			Resistant isolates		
Isolate	EC ₅₀ to myclobutanil	EC ₅₀ to penconazole	Isolate	EC ₅₀ to myclobutanil	EC ₅₀ to penconazole
1	0.07	0.03	5	1.28	1.65
2	0.11	0.04	6	1.49	0.94
3	0.30	0.10	7	2.04	1.09
4	0.23	0.09	8	11.35	1.14
Mean	0.18	0.07	Mean	4.04	1.21

Figure 1 Mean sensitivity of *Venturia inaequalis* isolates to two demethylation inhibitor (DMI) fungicides, myclobutanil and penconazole, and to dodine, ranked according to the concentration that inhibited mycelial growth by 50% (EC₅₀) for each fungicide. Open circles indicate organic orchards, black and grey circles indicate Integrated Fruit Production orchards, with black circles indicating orchards in which resistance management guidelines were exceeded. Numbers accompanying the dotted lines indicate national mean EC₅₀ (mg/litre) and sampling season from three previous New Zealand studies: 1990-91 (Whelan et al. 1992), 1993-94 (Bakker et al. 1995) and 1997-98 (Bakker 1999). Lines below each graph link orchard numbers with mean EC₅₀ values that are not significantly different (LSD $\alpha=0.05$), from ANOVA of log EC₅₀ values.

(1992), but was only slightly greater than the mean of 0.10 reported by Bakker et al. (1995). Previous Hawke's Bay EC₅₀ values for penconazole were 0.033 mg/litre (Whelan et al. 1992) and 0.11 mg/litre (Bakker et al. 1995).

Dodine sensitivity

For dodine, there was very little statistical separation between orchard mean EC₅₀ values and only the two orchards (8 and 10) with the least sensitive isolates were significantly different from the most sensitive orchards (Figure 1). However, as for the DMI fungicides, the isolates from organic orchards tended to have low EC₅₀ values and the isolates from orchards in which resistance management guidelines were exceeded tended to have higher EC₅₀ values. The overall mean EC₅₀ value of 0.24 mg/litre (Table 1) was lower than the values of 0.85 mg/litre reported by Bakker et al. (1995) and 0.527 reported by Bakker (1999).

DMI cross-resistance

Regression of penconazole log₁₀ EC₅₀ on myclobutanil log₁₀ EC₅₀ for individual isolates was very highly significant ($P < 0.001$), with an R^2 value of 0.56, indicating a high degree of cross-resistance between these two fungicides. There was no significant relationship between log EC₅₀ for dodine and log₁₀ EC₅₀ for either myclobutanil or penconazole ($P > 0.05$), indicating no cross-resistance between the DMI fungicides and dodine.

Sensitivity in relation to fungicide use

Orchard mean EC₅₀ values for myclobutanil were significantly correlated with the mean number of myclobutanil applications per season (Table 3), but there was no similar correlation for penconazole. Both myclobutanil and penconazole EC₅₀ values were correlated with total DMI fungicide use. Dodine EC₅₀ was also significantly correlated with use of the individual DMI active ingredients and with total DMI use, but, unexpectedly, not with dodine usage. This was clearly not the result of cross-resistance between DMIs and dodine, as explained above. The correlation between dodine EC₅₀ and DMI usage may have resulted from long-term fungicide use patterns in specific orchards that were not detected in the 4 years of spray diary data examined in this study.

Disease incidence in relation to fungicide sensitivity and fungicide use

For the DMI fungicides, no significant correlations ($P > 0.05$) were found between incidence of black spot on leaves or fruit and either orchard EC₅₀ values, or numbers of DMI fungicide applications per season. For dodine, there was a significant ($P < 0.05$) correlation ($r = 0.623$) between mean EC₅₀ and fruit black spot incidence, but not for leaf black spot incidence. Disease incidence was not correlated with numbers of dodine applications per season.

Table 3 Correlation coefficient (r) values between orchard mean EC₅₀ value for *Venturia inaequalis* for each of three fungicides and each of four fungicide usage categories, represented by the mean number of fungicide applications per season, for 15 Hawke's Bay apple orchards. The number of fungicide applications in each category was the 4-year average for the 2007-08, 2008-09, 2009-10 and 2010-11 seasons. Correlation coefficients in bold are significant ($P = 0.05$, 13 df).

Mean EC ₅₀	Fungicide usage category (mean no. applications/season)			
	Myclobutanil	Penconazole	Total DMI ¹	Dodine
Myclobutanil	0.629	0.019	0.714	0.796
Penconazole	0.447	0.144	0.670	0.715
Dodine	0.436	0.428	0.624	0.413

¹Demethylation inhibitor.

Table 4 Black spot severity, as mean percentage of leaf area affected, on potted apple plants inoculated with *Venturia inaequalis* isolates that were either sensitive or resistant to demethylation inhibitor fungicides and then sprayed with three fungicide treatments. Means accompanied by the same letter across the whole table are not significantly different (LSD $\alpha=0.05$).

Fungicide treatment	Sensitive isolates	Insensitive isolates
Water control	80.6 c	84.7 c
Myclobutanil	0.2 a	38.5 b
Flusilazol	0.0 a	1.1 a

Plant inoculations with isolates resistant and sensitive to myclobutanil

For the apple trees inoculated with the most DMI-sensitive *V. inaequalis* isolates, both myclobutanil and flusilazol almost completely inhibited black spot development (assessed as severity) compared with the water sprayed control (Table 4). For trees inoculated with the most resistant isolates, flusilazol gave a 99% reduction in black spot severity, from 84.7 to 1.1% severity, but myclobutanil reduced severity by only 55%, from 84.7 to 38.5%.

DISCUSSION

This Hawke's Bay study during 2010-2011 suggests that the trend for increasing resistance by *V. inaequalis* to DMI fungicides, which was reported to be occurring during the early 1990s by Bakker et al. (1995), has continued for myclobutanil. However, for penconazole there was less evidence of a decrease in sensitivity since the survey in 1993-94 by Bakker et al. (1995). In the present experiment, the EC_{50} values varied significantly among the orchards surveyed, for both myclobutanil and penconazole, and there was evidence that lower sensitivity occurred in orchards with higher use of DMI fungicides, particularly where resistance management guidelines had been exceeded. All the instances where DMI resistance management guidelines were exceeded in the 4 years of spray diaries examined were during the 2009-2010 season, which was particularly difficult for black spot control. Organic orchards tended to have lower EC_{50} values than most of the IFP orchards.

Understanding relationships between increases in EC_{50} values as resistance develops and loss

of disease control is crucial for understanding, predicting and managing resistance. In Italy, a mean EC_{50} value for penconazole of 0.106 mg/litre was found in an orchard with apple scab control problems (Fiaccadori et al. 1987). However, the association between EC_{50} and a disease control problem was based on observation, rather than disease control experiments, so its reliability was uncertain. In this study the plant inoculation experiment showed that selected myclobutanil-resistant strains were only partially controlled by myclobutanil. Given that these strains had a mean EC_{50} of 4.04 mg/litre for myclobutanil, which was much higher than any of the orchard mean values, the average degree of DMI resistance in Hawke's Bay orchards was unlikely to be having a noticeable impact on field disease control in the 2010-11 season. This was supported by the absence of correlations between DMI fungicide sensitivity and black spot incidence in the sampled orchards. However, strains of *V. inaequalis* that can grow in the presence of DMI fungicides are clearly present in Hawke's Bay orchards and loss of disease control efficacy could be expected in some field situations.

Agar plate tests, both in this and earlier studies, suggest that some isolates of *V. inaequalis* may have cross-resistance to different DMI active ingredients. However, the plant inoculation study, in which another DMI, flusilazol, gave complete control of myclobutanil-resistant *V. inaequalis* strains, suggested that individual DMI active ingredients may be less cross-resistant than agar tests indicated. If field control of black spot can still be obtained by carefully managing usage of specific DMI active ingredients, then DMI fungicides could continue to play an effective role even in the presence of developing resistance.

The continuing trend towards DMI resistance found in this study is worrying and suggests more careful management of DMI resistance risk is required. However, for the New Zealand Committee on Pesticide Resistance (Beresford et al. 2009) to develop more specific DMI resistance management guidelines, greater knowledge about in vitro fungicide sensitivity, cross-resistance and loss of disease control in apple orchards is required.

For dodine, there does not appear to have been a decrease in sensitivity of *V. inaequalis* in Hawke's Bay; EC₅₀ values in this study were relatively low compared with previous New Zealand studies (Bakker et al 1995, Bakker 1999). The absence of resistance development to dodine is encouraging, especially since dodine resistance has become widespread overseas. It suggests that the resistance management guideline of a maximum of three applications per season (Beresford 2005b) is helping to prevent resistance development.

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