Resistance of *Venturia inaequalis* to demethylation inhibitor and dodine fungicides in four New Zealand apple-growing regions

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Abstract A survey of 41 apple orchards in Hawke's Bay (25), Nelson (7), Otago (4) and Waikato (5) provided 796 isolates of *Venturia inaequalis* (black spot or scab), which were tested using an agar plate assay for sensitivity to two demethylation inhibitor (DMI) fungicides (myclobutanil and penconazole) and to dodine. Each fungicide was used at two concentrations to distinguish between highly sensitive, sensitive and resistant isolates. Sensitivity to DMIs in all regions was lower than baseline sensitivity in previous studies, particularly for myclobutanil. Waikato showed significantly lower DMI sensitivity than other regions. Dodine sensitivity was greater than in the 1990s, although Otago isolates were significantly less dodine-sensitive than those from other regions. In a plant inoculation assay to test control by five DMI fungicides of disease caused by resistant isolates, flusilazol and difenoconazole gave significantly better disease control of resistant isolates than myclobutanil, penconazole or fenbuconazole, at standard field rates.

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

INTRODUCTION

Fungicides with site-specific modes of action that are used in apple spray programmes for control of black spot (scab), caused by *Venturia inaequalis*, include demethylation inhibitor (DMI), dodine, quinone outside inhibitor (QoI) and anilopyrimidine (AP) groups. These fungicides are currently being investigated in relation to fungicide resistance development in *V. inaequalis* (Beresford et al. 2012; Viljanen-Rollinson et al. 2013; Larsen et al. 2013; FRAC 2013). The potential

impacts of resistance on the New Zealand apple industry include increased economic losses from increased incidence of black spot in orchards and threats to market access from diseased fruit and increased fungicide residues.

Current New Zealand DMI resistance management guidelines recommend a maximum of four DMI applications per season and mixing of each application with a fungicide from a different cross-resistance group (Beresford

2005a). For dodine, resistance management guidelines recommend a maximum of three applications per season and avoidance of consecutive applications (Beresford 2005b).

A survey of Hawke's Bay apple orchards in the 2010-11 season (Beresford et al. 2012) indicated that DMI sensitivity in *V. inaequalis* had decreased since surveys in the 1990s (Whelan et al. 1992; Bakker et al. 1995), to a degree where loss of black spot control could now be expected in some orchards. There was no evidence of a loss of sensitivity to dodine in Hawke's Bay since the 1990s (Bakker et al. 1995; Bakker 1999).

The present study undertook a wider survey to determine the frequency of DMI and dodine resistance in all the main apple-growing regions of New Zealand. Of the five DMI active ingredients currently registered for use on apples in New Zealand, myclobutanil and penconazole were chosen for testing because they have been widely used and their sensitivity has previously been monitored, both in New Zealand and overseas. All five DMI active ingredients were also tested for their ability to control black spot caused by selected sensitive and resistant *V. inaequalis* isolates on potted apple trees.

MATERIALS AND METHODS

Orchard sampling and *V. inaequalis* isolation

Populations of about 20 *V. inaequalis* isolates per orchard were obtained during December 2011 and January 2012 from apple fruit lesions from 41 orchard blocks (Table 1) in Hawke's Bay (25), Nelson (7), Otago (4) and Waikato (5). The cultivars sampled were 'Royal Gala' (35 blocks), 'Braeburn' (two blocks), 'Fuji' (two blocks) and Pink Lady® (two blocks). *Venturia inaequalis* conidia were scraped from the sporulating fruit lesions and streaked onto water agar. After 48 h of incubation at 20°C, individual germinating conidia were isolated onto potato dextrose agar (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 in natural light and were sub-cultured, if necessary, to eliminate contaminants. A total of 796 single conidium isolates was obtained.

Table 1 The 41 apple orchards in Hawke's Bay (HB), Nelson (Nel), Waikato (Wai) and Central Otago (Ot) from which populations of *Venturia inaequalis* were isolated during the 2011-2012 season for fungicide sensitivity testing.

Orchard no.	Production system ²	Cultivar ³	No. isolates
Hawke's Bay			
HB-1 ¹	IFP	RG	20
HB-2 ¹	IFP	RG	20
HB-3 ¹	IFP	RG	20
HB-4	IFP	RG	20
HB-5 ¹	Organic	Br	20
HB-6	Organic	Fu	20
HB-7	Organic	PL	20
HB-8	Organic	RG	20
HB-9	IFP	RG	20
HB-10 ¹	IFP	RG	20
HB-11	IFP	RG	20
HB-12	IFP	RG	20
HB-13 ¹	IFP	RG	20
HB-14	IFP	RG	20
HB-15 ¹	Organic	Br	20
HB-16 ¹	IFP	RG	20
HB-17	IFP	RG	20
HB-18	IFP	RG	20
HB-19	IFP	RG	20
HB-20	IFP	RG	20
HB-21	IFP	RG	20
HB-22	IFP	RG	20
HB-23	IFP	RG	14
HB-24	IFP	RG	19
HB-25	IFP	Fu	20
Nelson			
Ne-01	IFP	RG	8
Ne-02	IFP	RG	20
Ne-03	IFP	RG	20
Ne-04	IFP	RG	19
Ne-05	IFP	RG	18
Ne-06	IFP	RG	19
Ne-07	IFP	RG	19
Otago			
Ot-01	IFP	RG	20
Ot-02	IFP	RG	20
Ot-03	IFP	RG	20
Ot-04	IFP	RG	20
Waikato			
Wai-01	IFP	RG	20
Wai-02	IFP	RG	20
Wai-03	IFP	RG	20
Wai-04	IFP	PL	20
Wai-05	IFP	RG	20

¹Orchards also sampled in 2010-11 (Beresford et al. 2012).

²IFP=Integrated fruit production.

³RG='Royal Gala'; BR='Braeburn'; Fu='Fuji'; PL=Pink Lady®.

Mycelial growth assay

Fungicide sensitivity was determined using two discriminatory concentrations of myclobutanil (Sythane®), penconazole (Topas®) and technical grade dodine (Tables 2 & 3). The mycelial colony diameter of each isolate was measured on PDA Petri plates amended with each of the two concentrations. These discriminatory concentrations were chosen from the results of the Hawke's Bay survey in 2010-11 (Beresford et al. 2012), based on fungicide concentrations that inhibited growth by 50% (EC₅₀) and the degree of control by these fungicides of disease on apple leaves that were inoculated with resistant isolates. The upper discriminatory concentrations were chosen to be relatively high values that would ensure resistance frequency was not overestimated.

The fungicide-amended plates were inoculated in the centre with an agar disc from the edge of an actively growing colony. Two replicate plates were used for each isolate and fungicide concentration. The Petri plates were incubated for 42 days at 18-20°C and two diameters were measured on each plate. Sensitivity of each isolate was expressed as relative growth, which was the mean colony diameter on amended agar as a percentage of the diameter on the non-amended control.

Development of resistance to DMI fungicides generally appears as a quantitative decrease in sensitivity over time, and resistance is a relative

term. In this study, isolates with mean relative growth <50% of the non-amended control at the lower concentration were classified as sensitive. Isolates with mean relative growth ≥50% of the non-amended control at the lower concentration and ≤50% of the non-amended control at the upper concentration were classified as intermediate sensitivity. Isolates with mean growth at the upper concentration >50% of the control were classified as resistant.

Plant inoculation assay

The ability of five DMI fungicides to control DMI-resistant isolates of *V. inaequalis* was investigated on the leaves of 2-year-old potted 'Gala' apple trees. Shoots were inoculated with either a mixture of three resistant (R) isolates or a mixture of five sensitive (S) isolates (Table 4), which had been collected and characterised in the 2010-11 Hawke's Bay orchard survey (Beresford et al. 2012). After inoculation, a single spray application was made of eight fungicide treatments. They were (1) fenbuconazole, (2) fenbuconazole plus myclobutanil, (3) flusilazol, (4) difenoconazole, (5) myclobutanil, (6) penconazole, (7) water control (R) and (8) water control (S). Plants receiving treatments 1-7 had been inoculated with R isolates and those receiving Treatment 8 had been inoculated with S isolates: A ninth treatment of non-inoculated,

Table 2 Application rates and active ingredient concentration for five demethylation inhibitor (DMI) fungicides contained in six fungicide products registered for use against apple black spot in New Zealand. Sythane® and Topas® were used in the mycelial growth assay to test regional sensitivity of *Venturia inaequalis* isolates. All six products were used in the plant inoculation assay to investigate black spot control of DMI-resistant isolates.

Fungicide active ingredient (ai)	Product	ai in product (kg/kg or litre)	Product rate (g/litre) ¹	ai rate (g/litre) ¹
Fenbuconazole	Indar®	0.75	0.028	0.021
Fenbuconazole + Myclobutanil	Indar® Duo	0.75 + 0.40	0.028 + 0.090	0.021 + 0.036
Flusilazol	Nustar®	0.20	0.150	0.030
Difenoconazole	Score® 10 WG	0.10	0.250	0.025
Myclobutanil	Sythane® 400 WP	0.40	0.120	0.048
Penconazole	Topas® 200EW	0.20	0.125	0.025

¹Application rates for the plant inoculation assay

Table 3 Discriminatory concentrations of myclobutanil, penconazole and dodine used in the mycelial growth assay to determine the regional frequency of fungicide resistance in *Venturia inaequalis*.

Fungicide group	Active ingredient	Lower dose (mg/litre)	Upper dose (mg/litre)
DMI	Myclobutanil	0.20	1.00
DMI	Penconazole	0.10	0.50
Dodine	Dodine	0.20	1.00

non-fungicide-treated trees was used as a check for background *V. inaequalis* infection. Fungicide product details and application rates are shown in Table 2.

Potted trees with 5-10 leaves per shoot were used for the trial and were inoculated with 1×10^5 *V. inaequalis* conidia/ml, comprising equal mixtures of R or S isolates. Conidia for inoculation were produced from PDA cultures growing on a cellophane layer to promote sporulation. The youngest unrolled leaf at the time of inoculation was tagged so that inoculated leaves could be identified later. 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, as two shoots on each of three plants, were used for each treatment. After fungicide treatment, the trees were placed in a glasshouse in a randomised block design for 20 days then the five youngest inoculated leaves on each shoot were visually assessed for percentage of leaf area with sporulating black spot lesions.

Statistical analysis

Statistical analyses were done using Genstat version 8.1. ANOVA was used to identify significant treatment effects, with mean separation by Tukey's least significant difference (LSD, $P < 0.05$). Analysis 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 plant inoculation study did not require transformation.

Fungicide usage in the sampled orchards

Use of DMI and dodine fungicides in the sampled apple blocks was determined from the Pipfruit New Zealand Inc. spray diary database. The numbers of applications of products

containing myclobutanil, penconazole, other DMI fungicides and dodine were summarised for five seasons (2007-2008, 2008-2009, 2009-2010 and 2010-2011 and for the 2011-12 season up to the time the *V. inaequalis* samples were taken). Four of the organic orchards in Hawke's Bay had received no synthetic fungicides for at least 5 years, but organic orchard HB-06 had been under organic management for 3 years and had received synthetic fungicides before that.

RESULTS

Mycelial growth assay

The frequency of resistant isolates was higher for myclobutanil than either penconazole or dodine in most orchards from all regions (Figure 1). Every orchard, except for organic orchard HB-05, which had been under organic management for 12 years, had some myclobutanil resistance. The highest myclobutanil resistance frequencies ($\geq 50\%$) occurred in three Waikato orchards. These orchards also all had a consistent presence of penconazole resistance, but only two out of the five had dodine resistance. Some of the Hawke's Bay orchards had a relatively high penconazole resistance frequency ($> 20\%$), particularly HB-19, HB-22 and HB-25. In Otago, the myclobutanil resistance frequency tended to be lower than in other regions and only one Otago orchard had any penconazole resistance (Ot-02). However, all four Otago orchards showed slight dodine resistance. The Nelson orchards had variable myclobutanil resistance and only two out of the seven had any penconazole resistance. One out of the seven had some dodine resistance. Dodine resistance was sporadic in Hawke's Bay, with the organic orchard HB-06 having the highest frequency (40%). HB-06 also had a high frequency of myclobutanil and penconazole resistant isolates.

Table 4 EC₅₀ (mean fungicide concentration that inhibited mycelial growth by 50%; mg/litre) for myclobutanil and penconazole for five demethylation inhibitor (DMI)-sensitive and three DMI-resistant *Venturia inaequalis* isolates.

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	6	1.49	0.94
2	0.30	0.10	7	2.04	1.09
3	0.12	0.08	8	1.17	0.74
4	0.10	0.06			
5	0.33	0.07			
Mean	0.18	0.07	Mean	1.57	0.92

In relation to the two DMI fungicides, the orchards with a high myclobutanil resistance frequency did not necessarily have a high penconazole resistance frequency. However, for all the individual isolates pooled, the relative growth on myclobutanil and penconazole at the upper discriminatory concentration was highly correlated ($r = 0.70$; $P < 0.001$), indicating a high degree of cross-resistance between these two DMI fungicides.

Regional means of relative growth provided an indication of the degree of DMI-resistance in individual orchards and summarised the trends in resistance between regions (Figure 2). Waikato orchards had significantly lower sensitivity to both DMIs at both concentrations than orchards in other regions, which did not differ significantly. For dodine, Otago isolates had significantly lower sensitivity at the lower discriminatory concentration than isolates from

Figure 1 Resistance frequency, as percentage of *Venturia inaequalis* isolates per apple orchard with >50% relative growth at the upper discriminatory concentration of myclobutanil, penconazole or dodine. Forty-one orchards in Hawke's Bay, Nelson, Otago and Waikato, are ranked within each region by myclobutanil resistance frequency. The dotted line indicates a resistance frequency of 50%.

Figure 2 Regional means for relative growth of *Venturia inaequalis* at two discriminatory concentrations for each of the fungicides myclobutanil, penconazole and dodine from 41 apple orchards in Hawke's Bay, Nelson, Otago and Waikato. Relative growth is the colony diameter after 42 days on fungicide-amended agar as a percentage of the non-fungicide-amended control. Bars within each fungicide × concentration accompanied by the same letter are not significantly different (Tukey's LSD, $P < 0.05$).

Hawke's Bay or Waikato, and Nelson isolates were intermediate. There were no significant differences between regions at the upper dodine discriminatory concentration.

Fungicide sensitivity in relation to fungicide usage

The orchards with the highest resistance frequency to myclobutanil were not necessarily those with the highest DMI use (Figure 1 compared with Figure 3). Waikato orchards, which showed the greatest DMI-resistance, reported relatively low DMI use, which was similar to DMI use in Otago, where DMI resistance frequency was relatively low. The average use of DMIs in Waikato and Otago did not exceed the resistance management guidelines of four applications per season, unlike Hawke's Bay and Nelson, where seven out of 25 (28%) and five out of seven (71%) of orchards, respectively, had average usage that exceeded the guidelines.

Of the three Hawke's Bay orchards with high penconazole resistance frequency (Figure 1), average DMI use in two exceeded the resistance management guidelines (HB-19, 6.6 applications/season and HB-25, 7.2 applications/season) and

the third was the organic orchard HB-06, with no reported DMI use in the previous five years.

There was no overall correlation between DMI usage and either myclobutanil or penconazole resistance frequency or mean relative mycelial growth at either discriminatory concentration. For orchards that exceeded the DMI resistance management guidelines, there was a significant correlation between penconazole resistance frequency and number of DMI applications per season ($r = 0.35$, $P < 0.05$). However, this was driven by orchards HB25 and HB19, which had both high penconazole resistance frequency and high DMI usage.

Although there was no overall correlation between dodine resistance frequency and dodine usage, orchard HB-19, which exceeded the dodine resistance management guidelines, had a relatively high dodine resistance frequency. However, other orchards contradicted this trend, e.g. HB-25, which had no isolates classified as resistant but the highest dodine usage of all orchards, and organic orchard HB-06, which had no dodine usage recorded, but 10% dodine-resistant isolates.

Figure 3 Fungicide usage as mean number of demethylation inhibitor (DMI) or dodine applications per season over five seasons for 41 apple orchards in the 2011-12 resistance survey. Orchards are ranked by frequency of myclobutanil resistance within each region, as for Figure 1. The dotted lines indicate the maximum number of applications per season recommended in resistance management guidelines.

Plant inoculations with DMI-resistant isolates

The severity of black spot on leaves inoculated with R or S isolates then treated with different DMI fungicides, or water, fell into three statistically different groups (Figure 4). These were (a) little or no disease (flusilazol and difenoconazole), (b) intermediate amount of disease (fenbuconazole, fenbuconazole plus myclobutanil, myclobutanil and penconazole) and (c) maximum amount of disease (control trees inoculated with R or S isolates and not fungicide-treated). The non-inoculated, non-fungicide-treated control trees had zero leaf area affected by black spot at the end of the experiment, indicating there was no background *V. inaequalis* infection.

The trees not treated with fungicide showed no difference in disease between the R and S isolates, indicating that both types of isolates

were equally capable of causing disease. Although this experiment did not include S isolates treated with the fungicides, it is probable that all the DMI fungicides would have given a high degree of black spot control for sensitive isolates, as had been shown previously (Beresford et al. 2012).

DISCUSSION

The results of this study, when compared with previous New Zealand and overseas studies (Table 5), show that a substantial loss of sensitivity in *V. inaequalis* to DMI fungicides has occurred in all of New Zealand's major apple growing regions. Resistance to myclobutanil is very widespread and although resistance to penconazole was at a lower frequency, it was also present in many orchards. Waikato was the region with the greatest DMI resistance frequency. Although

Figure 4 Black spot severity as mean percentage of leaf area affected on potted apple plants inoculated with *Venturia inaequalis* isolates that were either resistant (R) or sensitive (S) to demethylation inhibitor fungicides and then sprayed with eight fungicide treatments. Background infection was checked using the not-inoculated, no-fungicide treatment. Means accompanied by the same letter are not significantly different (LSD $P < 0.05$).

this region has a high climatic risk of black spot, requiring intensive fungicide spray programmes, the high resistance frequency was not able to be correlated with 5-year DMI fungicide usage from the orchard spray diaries. There was a generally low correlation between DMI fungicide usage and muclobutanil or penconazole resistance in each region. This could be the result of representational error because of the relatively small *V. inaequalis* sample population from each orchard (20 isolates). It could also be the result of spray diary inaccuracy or inaccurate matching between spray diary records and the orchard areas actually sampled for *V. inaequalis*.

Only one orchard had no DMI resistant isolates: HB-05 had been in organic production with no DMI use for 12 years. Most of the other organic orchards also had low DMI resistance frequency, except HB06, which had been under organic production for only 3 years and was known to have had DMI and dodine applications prior to organic conversion. The widespread occurrence of myclobutanil resistance, even in some organic orchards, and the lack of correlation with 5-year DMI fungicide usage suggests that myclobutanil

resistance has been developing over a wide area for a long period.

The plant inoculation assay showed that for DMI-resistant strains of *V. inaequalis*, myclobutanil, penconazole and fenbuconazole gave lower disease control than the newer DMI active ingredients, flusilazol and difenoconazole. This has also been reported overseas (Rosenberger & Cox 2010). The product mixture containing both fenbuconazole and myclobutanil did not significantly improve disease control over either one of the active ingredients alone. While *V. inaequalis* is presumed to be cross resistant to all five DMI active ingredients, flusilazol and difenoconazole would be expected to provide more effective black spot control for the *V. inaequalis* strains currently present in New Zealand orchards than muclobutanil, penconazole or fenbuconazole. However, despite the presence of resistant isolates, myclobutanil could still be expected to give disease control in orchards where the frequency of DMI-resistant isolates is low.

The upper discriminatory myclobutanil concentration chosen for this study to classify *V. inaequalis* isolates as resistant was high compared with the discriminatory doses that are generally used (Russell 2002). Köller et al. (1997) and Rosenberger & Cox (2010) reported a discriminatory myclobutanil dose of 0.1 mg/litre in the USA and Canada, which was slightly lower than the lower discriminatory concentration used in the present study to identify sensitive isolates. The upper discriminatory concentration used in the present study was derived from agar-based EC_{50} values that were shown to be associated with lack of disease control on inoculated and DMI-treated apple plants. Discriminatory doses in other studies have been derived from shifts in sensitivity on agar above a baseline, sometimes without reference to actual disease control. DMI resistance is quantitative and even the isolates identified in this study as DMI-resistant at the upper discriminatory concentration on agar were partially controlled by both myclobutanil and penconazole. The high concentration thresholds for defining resistance

Table 5 Comparative values for the mean fungicide concentration that inhibits growth of *Venturia inaequalis* by 50% (EC₅₀) for two demethylation inhibitor (DMI) fungicides (myclobutanil and penconazole) and for dodine.

Reference	Year surveyed	Geography	EC ₅₀ (mg/litre)		
			Myclobutanil	Penconazole	Dodine
Köller & Wilcox (1999) ¹	1970s	USA & Canada	-	-	0.2
Köller et al. (1991) ²	ca 1990	NY State, USA	0.07	0.03	-
Jobin & Carisse (2007) ³	2003	Quebec	0.064	-	-
Jobin & Carisse (2007) ⁴	2003	Quebec	1.5-3.1	-	-
Whelan et al. (1992) ²	1990-91	NZ	0.16	0.039	-
Bakker et al. (1995) ²	1993-94	NZ	0.43	0.10	0.85
Bakker (1999) ²	1997-98	NZ	-	-	0.527
Beresford et al. (2012) ²	2010-11	Hawke's Bay, NZ	0.69	0.12	0.24
This study ⁵	2011-12	NZ	0.59	0.11	0.25

¹Discriminatory dose to detect sensitive isolates.

²Baseline sensitivity.

³Commercial orchards.

⁴Commercial orchards with disease control failure.

⁵Estimated from relative growth at two concentrations in this study and EC₅₀ values from Beresford (2012).

in the present study are likely to relate well to practical resistance (Köller et al. 1997) associated with disease control problems in the field. Use of too low a discriminatory dose runs the risk of overestimating resistance frequency with respect to loss of disease control. In relation to detecting penconazole resistance in this study, it is possible that the relatively low frequency of resistant isolates in orchards determined by the mycelial growth assay, yet the poor control of resistant isolates on potted plants, may indicate that the upper discriminatory penconazole concentration was too high.

Dodine resistance in *V. inaequalis* does not appear to be a problem in New Zealand apple orchards, although it was present in all regions, particularly Otago. The present study confirmed the Hawke's Bay data from 2010-11 (Beresford et al. 2012) that dodine sensitivity has not increased since the 1990s and may have actually decreased (Table 4). Given the widespread resistance to dodine found overseas (Chapman et al. 2011), it suggests that the relatively conservative resistance management guideline in New Zealand of three applications per season is helping to reduce dodine resistance development. Overseas, dodine

resistance was reported to decline significantly after 4 years (Michigan) or 13 years (NY State) of no dodine use after a serious resistance problem had previously been reported (Köller & Wilcox 1999).

The Pipfruit New Zealand Inc. spray diary data showed that there were many instances where resistance guidelines were exceeded, especially for DMIs. Continued adherence to the New Zealand resistance management guidelines is essential and these guidelines are being updated by the New Zealand Committee on Pesticide Resistance (Beresford et al. 2009) to accommodate the new findings from this study.

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