Sensitivity of *Neonectria ditissima* to carbendazim fungicide in New Zealand

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Abstract Carbendazim can be used by New Zealand pipfruit growers for control of European canker, caused by *Neonectria ditissima*. A total of 162 *N. ditissima* isolates were tested for carbendazim sensitivity using a mycelial growth assay on amended malt extract agar plates at concentrations from 0-2 mg/litre. Generally, isolates ceased growth at 2 mg/litre carbendazim, with 65 isolates ceasing growth at 1 mg/litre. The EC_{50} values calculated for 19 isolates ranged from 0.51 to 1.38 mg/litre. The data suggested that isolates ranged from sensitive (EC_{50}<1 mg/litre) to having intermediate resistance (EC_{50}=1-10 mg/litre carbendazim), based on comparison with other agar-based studies. Resistance in other fungi has been reported at carbendazim concentrations of >100 mg/litre. The isolates tested were mostly sensitive to carbendazim, but some showed a shift towards intermediate resistance. Mycelial growth tests at 2 mg/litre may be a useful tool to screen single spore isolates quickly for potential carbendazim resistance.

Keywords European canker, *Neonectria galligena*, *Nectria galligena*, benzimidazole.

INTRODUCTION

Carbendazim (methyl 2-benzimidazole carbamate) or MBC is a systemic benzimidazole fungicide. Carbendazim was first reported in 1973 and was developed by BASF, Hoechst (now part of Bayer) and Dupont (Marshall 2002). Carbendazim has been used for control of a broad range of diseases in horticulture, arable crops, vegetables and ornamentals including postharvest food storage, and has been used as a seed pre-planting treatment. The reported mode of action is by inhibiting the development of fungi, probably by interfering with spindle formation at mitosis, thereby affecting cell division (Marshall 2002). Carbendazim resistance is due to a single point mutation in the DNA, which does not affect fitness. Because of this, it remains in the population for decades, unlike other resistances that gradually disappear from the population (Koenraadt et al. 1992; Yarden & Katan 1993; Leroux et al. 2002; Thind 2012). In New Zealand, carbendazim may be applied in spring to control summer rots on apple. Some pipfruit growers may also use carbendazim postharvest, in autumn, for leaf scar protection against *Neonectria ditissima* (syn. *Neonectria galligena*, *Nectria galligena*), the causal agent of European

canker. Pipfruit New Zealand, the pipfruit industry body, does not recommend postharvest carbendazim sprays during autumn/winter because of the risk that fungicide resistance will develop in summer rot fungi. However, growers use carbendazim during autumn because of its high efficacy resulting from systemic movement within the plant. Growers are encouraged to mix carbendazim with captan to reduce the risk of resistance developing in *N. ditissima*. The New Zealand Committee for Pesticide Resistance (NZCPR) recommends that carbendazim use should be restricted to a maximum of two applications per year and that its use is confined to the blossom period (Beresford 2004). In New Zealand, growers may spray two to six fungicide applications during the leaf fall period, using a range of products including captan and copper-based fungicides.

European canker affects tree health and consequently can reduce productivity. Pruning to remove diseased wood is an effective but costly control method. Fungicide protection of leaf scars during leaf fall is recommended, and fungicides used in spring and summer against other diseases probably reduce infection by *N. ditissima*.

Resistance to carbendazim has been reported for over 80 pathogens including the following species that have pipfruit as hosts: *Fusarium* spp., *Monilinia* spp., *Venturia* spp., *Colletotrichum* spp., *Podosphaera leucotricha*, *Botrytis cinerea* and *Elsinoe* spp. (FRAC 2004). However, resistance of *N. ditissima* to carbendazim has not been widely studied. The study by Weber & Palm (2010) on apple storage rot fungi in Germany included only eight isolates of *N. ditissima*. In that study resistance to the benzimidazole fungicide thiophanate-methyl, which breaks down to carbendazim in the plant, was determined in four fungal pathogens. They found a uniform response of intermediate resistance to thiophanate-methyl, with EC_{50} values of 1.0-1.7 ppm (EC_{50} = fungicide concentration that inhibits growth by 50%). This, to our knowledge, is the only report on carbendazim sensitivity tests to the causal organism of European canker.

The aim of the present work was to develop a screening method and to determine sensitivity, in terms of EC_{50} values, to carbendazim for New Zealand *N. ditissima* isolates.

MATERIALS AND METHODS

The methods of Weber & Palm (2010) were followed using malt extract agar (MEA, Merck) amended with a range of carbendazim concentrations (0 to 4 mg ai/litre). The fungicide-amended plates were then inoculated with two 5-mm plugs (equidistant to the margin and centre of the 9 cm plate) taken from the margins of actively growing isolates on non-amended MEA. The source of carbendazim was the commercially available fungicide Prolific® (500 g/litre carbendazim in the form of a suspension concentrate; Fruitfed Supplies). Pathogen isolates were obtained from the Plant & Food Research culture collection, Havelock North, New Zealand (13 isolates), the ICMP culture collection (8 isolates) and new field samples (141 isolates) collected during April-June 2013 in the Tasman region of New Zealand. Isolates had been either single conidium or single ascospore isolates, with the exception of the ICMP isolates, where the type of isolation could not be determined.

Isolates were screened in a series of six experiments, with selected 'standard' isolates included in each experimental set. Inoculated fungicide-amended plates (prepared in duplicates) were incubated at 18-20°C under a light bank (Philips TLD 58W/840) with a 16-h photoperiod. Plates were completely randomised and stacked up to five plates high. Experiment 1 was a preliminary test defining the carbendazim concentrations used and excluded in all further analyses. In Experiment 2, 139 isolates were tested at the higher carbendazim MEA concentrations (0, 2, 3 and 4 mg/litre). In Experiments 3 and 4, 19 isolates were tested at seven and eight different carbendazim concentrations, respectively (Exp. 3: 0, 0.2, 0.4, 1, 2, 3 and 4 mg/litre; Exp. 4: 0, 0.2, 0.4, 0.6, 1, 1.2, 1.4 and 2 mg/litre). In Experiments 5 (108 isolates) and 6 (78 isolates), isolates were tested at 0.4, 1 and 2 mg/litre carbendazim.

In each experiment, there were duplicate plates per isolate, with two mycelial plugs per plate placed at equidistance to each other and the Petri dish margins. For each plate, the two

mycelial plugs were taken from two separate colonies (2-3 weeks old). Colony diameters were measured after 7 or 8 days of incubation. After 3 weeks of exposure on the fungicide-amended plates, the fungal plugs that did not grow were moved onto fresh (unamended) MEA plates to determine their viability (particularly those from fungicide-amended plates with the higher carbendazim concentrations). In Experiments 5 and 6, mycelial plugs that failed to grow after the 3 weeks of exposure on fungicide-amended plates were then moved onto untreated MEA or potato dextrose agar (PDA, Merck) plates, where they were incubated for a further 10 days. Excluding Experiment 1, six isolates (15.1, 18.1, 47.1, RS 140c, RS 133c, RS 370p4) were included in all experiments and an additional seven isolates (12.1, 12.2, 19.1, 45.1, 47.2, 48.1, ICMP144417) were included in Experiments 2, 3, 4 and 5 or 6.

Statistical analyses

The data from Experiments 3 and 4 were combined for analysis of the dose response for each isolate. To combine the data, each of the diameters was converted to a growth radius by subtracting 5 (the diameter of the initial plug) and dividing by 2. Then the radii were converted to a percentage of the mean radius of the control (0 ppm concentration) separately for each isolate and experiment. A Gompertz curve (Berger 1981) was then fitted to the % radius results for each isolate.

The Gompertz curve had four parameters, A, C, B and M, and was in the form: Y = A + C*EXP(*EXP(-B*(X-M))), where there are horizontal asymptotes at A and A+C, and there is a point of inflexion at X=M.

The EC_{50} values for each isolate were calculated as the concentration that gave 50% of the growth radius of the upper asymptote of the fitted dose-response curve. Because the control treatment did not always give the highest mean growth, the fitted upper asymptote values were usually slightly above 100%. The EC_{50} value was calculated as

$EC_{50} = M + \{LOG(-LOG(-0.50*A/C))\} / (-B)$

For the remaining experiments, isolates were grouped into presence or absence of growth at <1 mg/litre and <2 mg/litre. The statistical software GenStat 16th edition (VSN International) was used.

RESULTS

Results from preliminary Experiment 1 were used to determine the fungicide concentrations for the dose-response assays in Experiments 3 and 4. In Experiment 2, none of the 139 isolates tested grew at the carbendazim concentrations tested (2, 3 and 4 mg/litre).

The EC_{50} values for each of the 19 isolates tested in Experiments 3 and 4 ranged from 0.51 to 1.38 (Table 1). When graphed, data in Table 1 illustrated a normally-distributed population (data not shown). For selected isolates, individual graphs of observed and fitted Gompertz curve results are shown in Figure 1. The majority of EC_{50} values were within the range of 0.7 to 1.3 mg/litre carbendazim, and all growth generally ceased at 2.0 mg/litre. Therefore the remaining isolates were tested at 0.4, 1 and 2 mg/litre carbendazim concentration in MEA (Experiments 5 and 6). From the 162 isolates tested in those experiments, all grew at 0.4 mg/litre, 65 ceased growth at 1 mg/litre and again, similarly to results from Experiments 2 and 3, all isolates ceased growth at 2 mg/litre. Mycelial plugs that showed no growth on the fungicide-amended plates were moved after approximately 3 weeks' exposure onto unamended MEA or PDA plates. All plugs (676) except two resumed growth and established a sizeable colony within 10 days of incubation.

It is noteworthy that in all experiments a few isolates showed minimal growth (<2 mm) on the edge of the plug at 2 mg/litre. Some fungal isolates also colonised the original MEA plug. This was also observed, but to a lesser extent, at 3 and 4 mg/litre carbendazim. This sporadic growth, however, was very inconsistent among plugs, plates and experiments.

DISCUSSION

Experiments were highly reproducible (Figure 1). The ability of *N. ditissima* isolates to colonise the

Table 1 Estimated EC_{50} values (and 95% confidence interval limits) for mycelial growth of 19 *Neonectria ditissima* isolates on carbendazim-amended agar using data combined from Experiments 3 and 4 (19 isolates tested at seven and eight carbendazim concentrations, respectively (Experiment 3: 0, 0.2, 0.4, 1, 2, 3 and 4 mg/litre; Experiment 4: 0, 0.2, 0.4, 0.6, 1, 1.2, 1.4 and 2 mg/litre). Motueka isolates with the same main number came from the same lesion – the digit separates individual isolates from one lesion.

Location, year of isolation	Isolate ID	EC_{50}	95% C.I.
Auckland, 2010	RS 370 P4	0.74	(0.68, 0.80)
Pukekohe, 2007	RS 133 C	0.51	(0.48, 0.55)
Motueka, 2013	12.1	0.76	(0.72, 0.80)
Motueka, 2013	12.2	0.70	(0.65, 0.74)
Motueka, 2013	15.1	0.86	(0.80, 0.93)
Motueka, 2013	15.2	0.86	(0.80, 0.92)
Motueka, 2013	18.1	1.02	(0.97, 1.06)
Motueka, 2013	18.2	0.92	(0.83, 1.01)
Motueka, 2013	19.1	0.97	(0.90, 1.04)
Motueka, 2013	19.2	0.67	(0.56, 0.78)
Motueka, 2013	24.1	0.76	(0.71, 0.81)
Motueka, 2013	24.2	0.87	(0.83, 0.92)
Motueka, 2013	45.1	0.84	(0.77, 0.92)
Motueka, 2013	45.2	0.82	(0.73, 0.90)
Motueka, 2013	47.1	1.20	(1.13, 1.27)
Motueka, 2013	47.2	1.23	(1.17, 1.30)
Motueka, 2013	48.1	1.19	(1.13, 1.24)
Motueka, 2013	48.2	1.38	(1.31, 1.46)
Nelson, 2006	ICMP 14417	0.94	(0.88, 0.99)

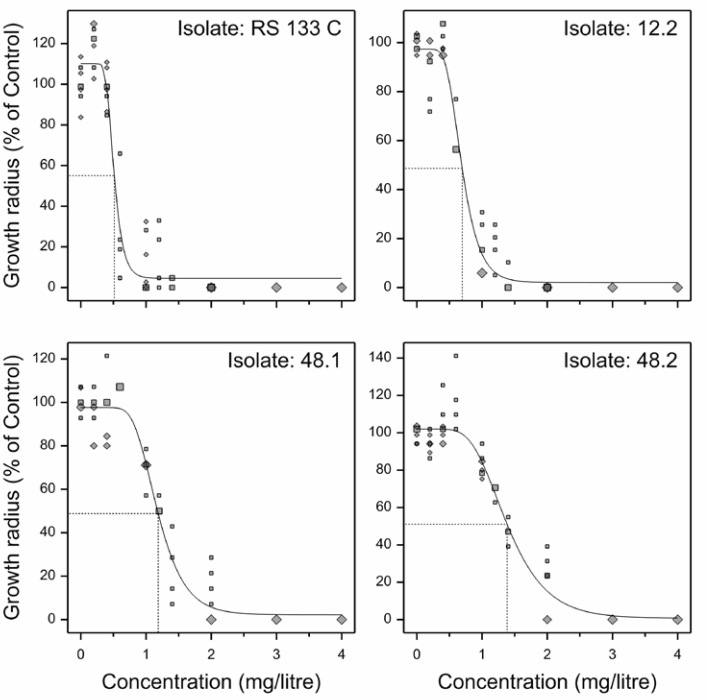

Figure 1 Observed and fitted Gompertz curve results for carbendazim-amended agar (concentration) on mycelial growth of *Neonectria ditissima* isolates. The symbol represents number of repeated observations (diamond = Experiment 3, square = Experiment 4). (19 isolates tested at seven and eight carbendazim concentrations, respectively (Experiment 3: 0, 0.2, 0.4, 1, 2, 3 and 4 mg/litre; Experiment 4: 0, 0.2, 0.4, 0.6, 1, 1.2, 1.4 and 2 mg/litre). Broken line shows EC_{50}.The curves for the lowest EC_{50} value (isolate RS 133 C), the highest (isolate 48.2) and two mid-range EC_{50} values are shown (isolate 12.2 and isolate 48.1).

original inoculum plug and the small (<2 mm) sporadic growth on fungicide-amended plates is attributed to slight variations in carbendazim concentrations among repeated plates and experiments, plus variations in inoculum plug depth, mycelial density and age. Carbendazim concentrations of 2-4 mg/litre also were fungistatic, with >99% of plugs resuming growth on unamended agar. This is in agreement with its reported mode of action (Manners 1993).

Weber & Palm (2010) reported intermediate resistance of *N. ditissima* to carbendazim at an EC_{50} value of 1.0–1.7. This compared with *Neofabraea perennans* and *N. alba* strains with carbendazim sensitivity (EC_{50}=0.07 mg/litre), intermediate resistance (EC_{50}=1.0–3.4 mg/litre) or high resistance (EC_{50}>400 mg/litre). All the measured EC_{50} values for *N. ditissima* in the present experiments were at or below these German results, ranging from 0.51 to 1.38 mg/litre.

The agar test identified relative differences in sensitivity between isolates. Approximately 40% of the New Zealand isolates studied were in the sensitive group (EC_{50}<1 mg/litre carbendazim), with the remaining 60% being in the intermediate resistance group (EC_{50}=1-10 mg/litre carbendazim) based on the proposed grouping of Weber & Palm (2010). This intermediate resistance of *N. ditissima* to carbendazim is similar to the intermediate resistance reported for *Monilinia fructicola* (Yoshimura 2004), *Venturia inaequalis* (Katan et al. 1983), *Botyris cinerea* (Koenraadt et al. 1992; Yardan & Katan 1993; Leroux et al. 2002), *Neofabrae perennans* and *N. alba* (Weber & Palm 2010), but was at the lower threshold of intermediate resistance; all isolates tested ceased growth at 2 mg/litre carbendazim. Indeed the data in Table 1 and the normally-distributed population indicate that there is no compelling evidence for two groups of isolates with different carbendazim responses.

The carbendazim concentrations used in the *in vitro* mycelial growth assays were a fraction (in multiples of ten to hundred) of those used in field applications for disease control of pathogens (recommended carbendazim field rate in apple is 125 mg active ingredient/litre for the fungicide Prolific®). No distinctly resistant (EC_{50}=10-100 mg/litre or greater) isolates were found: all isolates were inhibited at 2 mg/litre carbendazim. Field studies in Motueka confirmed the efficacy of carbendazim to protect leaf scars (Scheper & Stevenson 2011) as well as budding and heading back wounds from infection with *N. ditissima* (Scheper & Stevenson 2012, 2013). This suggests that New Zealand *N. ditissima* pathogen populations are sufficiently sensitive to benzimidazole fungicides for such fungicides to still provide field disease control using field-collected inoculum. A direct comparison between *in vitro* efficacy and field efficacy is difficult, therefore additional plant assays would be required to determine the actual control efficacy of benzimidazole fungicides of intermediate-resistant carbendazim *N. ditissima* isolates. Pipfruit New Zealand recommends avoiding postharvest use of carbendazim during autumn/winter to avoid selecting for possible resistant mutations that may be produced during the sexual cycle of *N. ditissima* (and other fungal pathogens, e.g. *N. alba* and *C. accutatum*).

Without published baseline data in New Zealand or elsewhere for comparison (Weber & Palm 2010), the sensitivity grouping observed here may be a natural distribution or may be an actual shift in *N. ditissima* isolates towards resistance development. Highly resistant pathogen strains have been associated with point mutations in the beta tubulin gene. Mutations at codons 198 and 200 may give rise to different levels of resistance – as observed in many other fungal pathogens (Koenraadt et al. 1992; Yarden & Katan 1993; Leroux et al. 2002; Thind 2012). Narrowly delimited EC_{50} values and discrete sensitivity groups indicate target gene mutations, whereas multidrug resistance is expected to give rise to a more varied range of EC_{50} values (Deising et al. 2008). Graphing data in Table 1 indicated a normally-distributed population. In this present study of 19 isolates, a range of sensitivities was observed. In the absence of baseline data and genetic testing for beta tubulin mutations, it cannot be ascertained if the carbendazim response is natural or is part of a multidrug resistance development. For example, multidrug resistance of *V. inaequalis* to demethylation inhibitor and dodine fungicides in apple has been reported (Beresford et al.

2013). It is suggested that mycelial growth tests at 2 mg/litre may be a useful tool to screen single spore isolates quickly for potential carbendazim resistance development in future monitoring.

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