SOFT FUNGICIDES FOR THE CONTROL OF *BOTRYTIS CINEREA* *IN VITRO* AND *IN VIVO* ON KIWIFRUIT

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ABSTRACT

Three soft fungicides were evaluated for the control of *Botrytis cinerea* on kiwifruit in laboratory and field experiments. Four isolates of *B. cinerea* were tested against two standard fungicides (carbendazim and sulphur) and three products: Mycosin (Schaette) and Mycosan (Schaette), commercially available BIOGRO certified fungicides based on diatomaceous earth, and chitosan (Sigma), derived from chitin (arthropod exoskeletons). Mycosin and Mycosan had good efficacy against *B. cinerea* in *in vitro* experiments, but failed to significantly reduce disease incidence *in vivo*. Chitosan showed limited efficacy against *B. cinerea* in *in vitro* experiments and had no significant effect *in vivo*.

Keywords: fungicides, kiwifruit, *Botrytis cinerea*, induced resistance.

INTRODUCTION

The widespread selection of fungicide resistant strains of *Botrytis cinerea* Persoon in New Zealand kiwifruit orchards has led to a reduced efficacy of preharvest fungicide applications for the control of botrytis storage rot (Manning and Pak 1995). Together with the advent of the KiwiGreen programme for the production of low residue fruit, reduced efficacy of traditional fungicides has stimulated the search for alternatives to conventional fungicides for disease control in kiwifruit.

Three naturally-based products were investigated to determine their potential for the control of *B. cinerea* in kiwifruit orchards. These were Mycosin (Schaette) and Mycosan (Schaette) which are commercially available BIOGRO certified fungicides based on diatomaceous earth. Mycosin (no added sulphur) and Mycosan (with 41% sulphur) are two naturally based, commercially available fungicides which are BIOGRO registered. Information supplied by the manufacturers New Zealand agent (Sustain-Ability) indicates that these products are considered to induce resistance within the host plant.

The third product tested was chitosan (Sigma), a β -1,4 glucosamine polymer which is a component of fungal cell walls. It is obtained commercially from the deacetylation of chitin obtained from arthropod exoskeletons. Chitosan has fungicidal activity against *B. cinerea* *in vitro*, as well as inducing resistance to fungal infection within plant tissues (Wilson *et al.* 1994).

The extent to which these products act as an inducer of disease resistance can be investigated by comparing *in vitro* and *in vivo* efficacies. Good *in vivo* efficacy with little or no *in vitro* efficacy would indicate that the compound plays a role in inducing disease resistance within the host plant.

The aim of this research was to investigate the potential of three naturally-based products (Mycosin, Mycosan and chitosan) as alternatives to standard fungicides. The efficacy of these products was investigated both *in vitro* and *in vivo*, and compared with untreated controls and standard fungicide treatments.

MATERIALS AND METHODS

In vitro evaluation

The effects of fungicides on conidial germination, germ tube length and mycelial growth rate were tested. Three different product rates (0.1, 1 and 10 g/litre) of each fungicide (except for Chitosan) were used. The products were incorporated into malt extract agar (MEA - Oxoid) by suspending the required quantity of product in sterile distilled water which was then added to the cooled medium. Unamended MEA was used as a control. Due to the low solubility of chitosan the product rates tested were 0.01, 0.1 and 1 g/litre. Product rates rather than a.i. were used as two of the compounds contained several active ingredients.

The fungicides tested were: 1) Delsene 75 DF (Du Pont) = carbendazim, a benzimidazole; 2) Thiovit (Sandoz) - 80% sulphur in the form of water soluble granules; 3) Chitosan (practical grade, Sigma), a β -1,4 glucose amine polymer (66% deacetylated); 4) Mycosan (Schaette) consisting of diatomaceous earth, sulphuric basalt, silicic acid, *Equisetum* (horsetail) extracts and 41% elemental sulphur; 5) Mycosin (Schaette) consisting of similar components to Mycosan, but with no sulphur.

A benzimidazole was chosen to represent the most commonly applied fungicide (benomyl) in the 1995 season. Sulphur was included to determine if any difference in efficacy between Mycosan and Mycosin could be attributed to the sulphur content of the former.

Four strains of *B. cinerea* were selected from kiwifruit fungicide resistance samples tested in this laboratory to provide a range of isolates with different fungicide resistance characteristics: 8849 (benzimidazole resistant, dicarboximide resistant), 8081 (benzimidazole sensitive, dicarboximide resistant), 8836 (benzimidazole resistant, dicarboximide sensitive) and 8890 (benzimidazole sensitive, dicarboximide sensitive). These isolates have been deposited with the International Collection of Micro-organisms from Plants (Landcare, Auckland).

The proportion of germinating conidia was determined by inoculating plates (3 replicates for each strain and product rate) of MEA with 50 μ l of a conidial suspension (2×10^5 conidia/ml) of each strain, and incubating in the dark at 15 °C for 19 hours. Conidial suspensions of each isolate were prepared by washing plates of 10 day old cultures grown on MEA at 20 °C under UV light with 5 ml of Tween 80 solution (0.1 ml/litre in sterile water). The washings were then combined and the spore concentration adjusted to 2×10^5 conidia/ml. The proportion of germinated conidia (germ tube length greater than the length of the conidia) in a sample of 100 conidia per plate was determined by microscopic examination of the inoculated plates after 19 hours.

Germ-tube length was measured on a sample of 30 germinated conidia per plate. Mycelial growth rate was determined by inoculating plates (3 replicates for each strain and product rate) with 5 mm mycelial plugs taken from the margin of actively growing colonies. Plates were then incubated at 20 °C in the dark for 5 days. The extent of the colony margin was marked after 2 and 4 days, to determine growth rates in mm/day.

Field evaluation

A field experiment was set up on a commercial kiwifruit property in South Auckland using a randomised block design. Twenty single vine plots were laid out in two rows of vines in a 0.35 ha block. Rows of treatment vines were separated by a single row of buffer vines. Treatment vines were separated within rows by two buffer vines. There were five treatments with four replicates. The treatments applied were: 1) water-sprayed control; 2) conventional spray programme - benomyl (Benlate, Du Pont) @ 0.5 g/litre; 3) Mycosan @ 10 g/litre; 4) Mycosin @ 10 g/litre; 5) Chitosan @ 1 g/litre.

A total of 4 sprays were applied over a 7 week period using a Massotti spray lance and Comet P48 pump at a rate of 1500 litres/ha. Applications dates were 27 November 1995 (mid-bloom), 7 and 18 December, 1995 (post-bloom), and 18 January 1996. For the benomyl treatment, only the first application was of benomyl, the remainder were water sprays in order to keep within label recommendations.

Disease incidence was assessed on flower petals (100 petals/plot) 3 days after the first treatment was applied. Petals were collected, transferred to the laboratory and incubated under humid conditions for 24 hours at 20 °C. Using a microscope, the petals were then examined for production of conidia which indicated infection with *B. cinerea*. Disease incidence on necrotic leaf tissue was determined on two occasions (late January and immediately prior to harvest in May). Necrotic leaf tissue was sampled by collecting 10 mm discs (50 discs/plot) which were then transferred to the laboratory and incubated under humid conditions for 24 hours at 20 °C. Leaf discs were then examined for production of conidia. The January necrotic leaf sample was collected one week after the final treatment application. The incidence of botrytis stem-end rots was assessed in fruit after 20 weeks cool-storage at 0 °C (6 trays of 36 count fruit per plot).

Analysis

Treatment effects from the laboratory experiments were assessed on the combined data from the four strains using ANOVA. There was no significant differences between the stains in response to the different treatment rates other than for the carbendazim treatment. Data from the field experiment was subjected to a two way ANOVA with treatment and block effects.

RESULTS

The effect of three different concentrations of each of the treatments on conidial germination is shown in Figure 1. Mycosan and Mycosin both significantly ($P < 0.001$) reduced conidial germination relative to the untreated control (Table 1), but only at the highest rate tested. There was no significant effect on the proportion of germinating conidia at any concentration of sulphur, carbendazim or chitosan.

FIGURE 1: Conidial germination rate (%) of *B. cinerea* at three concentrations of each treatment. Error bars indicate the mean standard error.

The effect of the treatments on the length of the germ tube is shown in Figure 2. All the treatments except chitosan significantly ($P<0.001$) reduced germ tube length at the lowest rate tested (0.1 g/litre). For chitosan, a significant ($P<0.001$) reduction was only achieved at the highest rate tested (1 g/litre), with a similar efficacy as sulphur (Table 1). The greatest reduction was obtained with Mycosan and Mycosin (Table 1). The effect of carbendazim varied according to the fungicide resistance characteristics of the isolates tested as illustrated by the standard errors in Figure 2.

FIGURE 2: Germ tube (mm) of *B. cinerea* at three concentrations of each treatment. Error bars indicate the mean standard error.

TABLE 1: Maximum efficacy of treatments relative to the unamended control on conidial germination, germ tube length and mycelial growth rate of *B. cinerea*.

Treatment	Germination rate (%)	Germ tube length (mm)	Growth rate (mm/day)
Sulphur	0.0	36.8	7.0
Carbendazim	13.8	76.8	74.7
Mycosin	80.1	92.6	91.5
Mycosan	81.2	90.5	95.6
Chitosan	2.4	37.9	56.0

The main effect of chitosan was on mycelial growth rate (Table 1). The effect of the different treatment concentrations on mycelial growth rates is shown in Figure 3. Mycosin and Mycosan both significantly ($P<0.001$) inhibited mycelial growth at the 10 g/litre rate. Again, the effect of carbendazim varied according to the fungicide resistance characteristics of the isolates tested, as shown by the standard errors in Figure 3. Sulphur did not significantly reduce mycelial growth, even at the highest rate tested.

FIGURE 3: Mycelial growth rates (mm/day) of *B. cinerea* at three concentrations of each treatment. Error bars indicate the mean standard error.

In the field experiment the incidence of *B. cinerea* on female petals was significantly ($P<0.05$) reduced with a single application of benomyl (Table 2). None of the other treatments significantly reduced the incidence of *B. cinerea* at flowering. Incidence of *B. cinerea* on discs of necrotic leaf tissue early in the season (6 weeks after fruit set) after four applications was similar for the Mycosin, Mycosan and Chitosan treatments, all of which were significantly less ($P<0.05$) than the benomyl treatment, although they did not differ significantly from the control. However, at the end of the season, the incidence of *B. cinerea* in the Mycosan treated plots was significantly ($P<0.05$) greater than the other treatments. The incidence in the benomyl and Mycosin treatments was also significantly ($P<0.05$) greater than the control.

There was no significant difference in the incidence of stem-end rots after 20 weeks cool-storage between any of the treatments (Table 2). All of the treatments had a high incidence of rots indicating that the vines had been subjected to high disease pressure.

TABLE 2: Incidence (%) of *B. cinerea* after treatment with fungicides on female petals (December), leaf discs (January and May) and stem-end rots after 20 weeks cool-storage at 0°C.

Treatment	Female petals	Leaf discs		Stem-end rots
		January	May	
Control	6.8 a	21.7 a,b	4.8 a	61 a
Benomyl	1.8 b	31.5 b	11.7 b	44 a
Mycosin	7.3 a	15.6 a	12.6 b	50 a
Mycosan	8.8 a	12.2 a	18.9 c	49 a
Chitosan	4.2 a,b	14.6 a	4.3 a	53 a

Values with letters in common do not differ significantly at $P < 0.05$.

DISCUSSION

Mycosin and Mycosan had good efficacy against *B. cinerea* *in vitro*, and were the only products which inhibited germination. Becker and Straub (1993) showed that Mycosin also markedly inhibited germination of *Venturia inaequalis*. The sulphur component of Mycosan is unlikely to have contributed significantly to its fungicidal activity as evidenced by the relatively poor efficacy of sulphur against *B. cinerea*.

However, there was no significant efficacy in the field experiment, suggesting that under conditions of high disease pressure these products are ineffective at inducing resistance to *B. cinerea* in kiwifruit vines. This contrasts with studies on other target organisms which have shown that these products are effective *in vivo*. Färber *et al.* (1990) obtained an efficacy for Mycosan on grapevines of 58% relative to an untreated control against downy mildew (*Plasmopora viticola*) and 88% against powdery mildew (*Uncinula necator*). For these two diseases, Häseli (1996) demonstrated that under Swiss conditions, Mycosan was almost as effective as the standard copper and sulphur sprays. In Germany, Mycosan has also been shown to be a viable alternative to copper in the control of apple blackspot (*Venturia inaequalis*) (Kienzle and Straub 1991) and in Switzerland, Mycosan gave comparable or better control of blackspot than sulphur (Bosshard *et al.* 1994).

A possible explanation for the lack of efficacy *in vivo* in this study was that only four applications were made, rather than the fortnightly applications as suggested on the label. At the least, this would suggest that any induced resistance is relatively short-lived. While the fungicidal activity demonstrated *in vitro* should provide good protectant activity where fortnightly applications are used, it must also be noted that even after only four applications, disease levels had increased in the treated plots at the end of the season, most probably as a result of phytotoxic effects. Kienzle and Straub (1991) found that 10 g/litre Mycosan caused more russetting of apples than sulphur applied at 5 g/litre.

Chitosan provided only mediocre fungicidal activity *in vitro*. However, if this product were an effective inducer of host plant resistance then it should be more effective *in vivo*. In the field, although the incidence in the chitosan-treated plots was lower than for some of the other treatments, it did not differ significantly from the control.

Chitosan has been used successfully as a post-harvest treatment, where it forms a protective layer on the surface of the fruit, improving the storage life and reducing the incidence of postharvest diseases (El Ghaouth *et al.* 1994). Treatment of fruit with chitosan is also believed to induce resistance by promoting the production of chitinases, which attack the chitin in the cell wall of the invading fungus (Wilson *et al.* 1994). In laboratory studies, El Ghaouth *et al.* (1994) found that chitosan was most effective at 6 g/litre, whereas in this study chitosan was applied at 1 g/litre due to its low water solubility. Chitosan is readily soluble in dilute organic acids, which allows higher rates to be obtained in laboratory scale experiments, but is impractical for large scale field trials.

The benzimidazole fungicides (carbendazim and benomyl) included as standard treatments gave variable results. In the laboratory excellent efficacy on germ tube and mycelial growth rate was obtained at low concentrations against sensitive isolates, although there was no inhibition of germination as occurred with the diatomaceous earth products. These products had the advantage of being effective against both sensitive and resistant strains. The effect of benomyl in the field experiment was consistent with the laboratory experiments. The initial application significantly reduced disease levels, but was followed by a stimulation of the *Botrytis* population. This is most likely due to an increase in the level of benzimidazole-resistant isolates resulting from the benomyl application. Pak and Manning (1995) showed that a single benomyl application results in a rapid build-up of fungicide resistant populations in kiwifruit orchards.

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