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Control of downy mildew of grapevines by boosting their natural defence system



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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CONTROL OF DOWNY MILDEW OF GRAPEVINES BY BOOSTING THEIR NATURAL DEFENCE SYSTEM

A final report to the Grape and Wine Research and Development Corporation

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1 Abstract 1
2 Executive summary 2
3 Introduction 4
4 Screening experiments 5
4.1 Introduction 5
4.2 Materials and methods: 5
4.2.1 Plant material 5
4.2.2 Screening trials 1 – 6 5
4.2.3 Screening Trial 7, *Trichoderma* 8
4.2.4 Data analysis 9
4.3 Results and discussion: 9
4.3.1 Initial screening 9
4.3.2 The effect of inoculum concentration 10
4.3.3 Trichoderma as an inducer of resistance against downy mildew 12
4.4 Conclusions 14
5 Characterising elicitor activity 16
5.1 Introduction 16
5.2 Materials and Methods 16
5.2.1 Brotomax® dose-response 16
5.2.2 Chitosan dose-response 16
5.2.3 Post-inoculation activity against downy mildew 16
5.2.4 Synergy of inducer treatments with fungicides 16
5.2.5 Activity on flower tissue against downy mildew 17
5.3 Results and discussion 17
5.3.1 Brotomax® dose-response relationship 17
5.3.2 Chitosan dose-response 18
5.3.3 Timing of elicitor application 19
5.3.4 Synergy between inducers and copper oxychloride 20
5.3.5 Antifungal activity on flower tissue 21
5.4 Conclusions 21
6 Mode of action of elicitors 23
6.1 Introduction 23
6.2 Materials and methods 23
6.2.1 Activity against zoospores 23
6.2.2 Systemic and translaminar activity 23
6.2.3 PR-protein hydrolytic enzyme activities 24
6.3 Results and discussion 24
6.3.1 Activity against zoospores 24
6.3.2 Systemic and translaminar activity 24
6.3.3 Pathogenesis related protein hydrolytic enzyme activity assays 25
6.4 Conclusions 26
7 Performance of elicitors in the field 27
7.1 Introduction 27

7.2 Materials and methods 27
7.2.1 Trial design 27
7.2.2 Inoculation 27
7.2.3 Disease assessment 28
7.3 Results and discussion 28
7.3.1 Downy mildew on inflorescences 28
7.3.2 Downy mildew control on leaves 29
7.3.3 Botrytis on bunches 29
7.4 Conclusions 30
8 Conclusions and Recommendations 31
Appendix 1. References 33
Appendix 2: Comparison of approximate fungicide costs 35
Appendix 3: Staff 36

1 Abstract

Fourteen potential elicitors of natural disease resistance were screened for activity against downy mildew on grapevines. Three of these products, Brotomax®, $AlCl_3$ and chitosan had high levels of efficacy against downy mildew. It was not proven conclusively that these three products activate host defence responses. In contrast to most inducers of plant defence, Brotomax®, $AlCl_3$ and chitosan all had direct antifungal activity against zoospores of the pathogen. Brotomax® and $AlCl_3$ also appeared to have some systemic activity while chitosan did not. Contact fungicide activity appeared to be responsible for most of the disease control observed. Both Brotomax® and chitosan treatments were trialed in the field where they provided equivalent protection to the standard protectant fungicide (copper oxychloride) on leaves but had lower efficacy than the standard treatments on developing bunches. All products tested showed no post-infection activity and must therefore be used before infections become established.

Brotomax® is currently marketed in Australia as a plant nutrient for grapevines. However, at the dose rates required for downy mildew control, it would be approximately six times more expensive than current synthetic fungicides. This high cost would be a major barrier to the adoption of Brotomax® as a fungicide product. Chitosan is a non-toxic and biodegradable product produced from crustacean waste. While chitosan-containing crop protection products are available in Australia, they are not registered as fungicides and at current prices, would not be economically viable for downy mildew control. An Australian potassium bicarbonate product (Ecocarb®) was found to have high levels of activity against downy mildew in the glasshouse in a trial conducted late in the project but further trials were not conducted due to time constraints.

2 Executive summary

Stimulating a plant's own defence mechanisms to protect against disease is a relatively new concept in plant protection. If this mechanism can be exploited effectively we may be able to greatly reduce our reliance on chemical pesticides in future. The first effective plant defence activators or 'elicitors' are now commercially available overseas for use in some crops. The aim of this research was to evaluate the potential of elicitors of plant defence for the control of downy mildew on grapevines in Australia.

Potential elicitors of plant defence, identified through literature searches and products currently available in the marketplace claimed to boost plant defence, were screened for efficacy against grapevine downy mildew. Only three of the products screened, Brotomax®(a nutrient formulation with claimed plant defence activator activity, currently marketed for viticultural use in Australia), chitosan and $AlCl_3$, had high levels of efficacy against downy mildew. $AlCl_3$ may be unsuitable for use as a fungicide as it was phytotoxic to the grapevines at efficacious doses and likely to pose environmental problems similar to copper. Several compounds claimed to be active against grapevine downy mildew in the scientific literature did not give high levels of disease control in this research.

All of the effective products identified in this study were found to have contact fungicide activity. They were able to prevent zoospore release from sporangia and were toxic to motile downy mildew zoospores. Some systemic protection on unsprayed leaves on treated plants was observed for Brotomax® but not chitosan. Antifungal activity was shown not to be cultivar specific with high levels of activity on both Chardonnay and Cabernet Sauvignon varieties. No post-inoculation activity was observed for Brotomax® or chitosan which is consistent with protectant fungicide activity or induction of host plant defences. No synergistic effect was observed when combining low doses of either Brotomax® or chitosan with a low rate of copper oxychloride. Both chitosan and Brotomax® were shown to work on both foliage and flower tissue and had similar efficacy against downy mildew under field conditions as they had in glasshouse experiments. Chitosan and Brotomax® were also tested for anti-fungal activity against *Botrytis cinerea*, the cause of Botrytis bunch rot, but no disease control was observed in the field trial.

The systemic fungicide options available to growers for downy mildew control are limited by the restriction of phosphorous acid to one spray per season, due to phosphite residues, and the resistance risk of phenylamide fungicides (a major problem in Europe and recently detected in Australia). With growers becoming increasing reliant on protectant fungicides, it is important to have environmentally friendlier options than the widely used copper formulations which can accumulate to toxic levels in soil with long term use and dithiocarbamates which are toxic to populations of beneficial mites (Beranard *et al.* 2001). Other more environmentally acceptable protectant fungicides such as the strobilurin group are also at risk of resistance developing. Clearly there is a need to develop more sustainable control practices for grapevine downy mildew.

While both Brotomax® and chitosan had good efficacy against downy mildew under field conditions, the use of either product in a commercial situation (assuming registration as plant protection chemicals) will depend on the pricing of the products. At current pricing, Brotomax® would not be economically viable at the dose rates required to give similar levels of efficacy as current fungicides such as copper compounds. However, Brotomax® is currently marketed as a nutrient formulation and application for this purpose at a 1% concentration could be expected to provide effective control of downy mildew and may eliminate the need for a downy mildew fungicide application. While there are unregistered chitosan containing products available in Australia, they are marketed for high value hydroponic applications and would be too expensive to be routinely used for downy mildew control in viticulture.

Chitosan is currently only commercially produced from crustacean waste and squid bone. Research into producing chitosan from alternative sources such as fungal cell walls may lead to cheaper chitosan in future. Recent Australian research to evaluate the potential of chitosan to enhance plant defence in agriculture has shown promising results for powdery mildew control in tomatoes (Walker *et al.* 2004). This report recommended the further development of chitosan as a crop protection product, especially in Solanaceae crops. Thus, there is potentially a large market and economies of scale if an agricultural chitosan were to be registered and marketed in Australia.

Another way the cost of treatment could be reduced is through different application technology such as recirculating sprayers. If the efficiency of the spray deposition process could be sufficiently increased, more expensive but environmentally friendly crop protection products such as chitosan could be routinely used.

A potassium bicarbonate formulation (Ecocarb®) was tested against downy mildew after learning of anecdotal evidence of efficacy against downy mildew (D. Madge *pers com*). This product showed promising results in a glasshouse trial. Because this experiment was conducted just before the end of the research project, no further studies were conducted with this compound. However, as this compound product is non-toxic, comparable in price to other fungicides, IPM compatible and already has registration for grapes for control of powdery mildew, further developmental work with this product is recommended to determine if commercially acceptable disease control can be achieved in the field.

3 Introduction

For over 100 years, researchers have known that plants can be pre-conditioned to resist diseases. The basis for this 'acquired physiological immunity' was unknown at the time and it wasn't until the 1960's when the concepts of local acquired resistance (LAR) and systemic acquired resistance (SAR) were demonstrated by showing Tobacco mosaic virus infected tobacco was protected from subsequent challenge inoculations with the virus. Since these early experiments, SAR has been shown to operate in numerous plant species against not only viral pathogens, but also fungi, bacteria and even herbivores. The discovery that certain chemical compounds could mimic the effect of pathogens in activating the SAR pathway opened the way for the development of activators or 'elicitors' of SAR as plant protection products. This led to the concept of induced resistance as a disease control strategy, utilising the plant's own defence mechanisms instead of applying chemicals to kill or slow the growth of pathogenic organisms. With increasing concern about pesticide use worldwide, due to environmental problems, potential effects on human health and pathogens developing resistance to chemicals, plant protection strategies less reliant on pesticide application are becoming increasingly attractive.

Salicylic acid (SA) is naturally produced by plants and is known to be involved in the SAR biochemical pathway. It has been demonstrated that exogenous application of SA can also activate the resistance pathway in plants (Ryals *et al.* 1996). The first plant defence activators or 'elicitors' developed commercially were structural analogues of SA. These compounds were able to provide long lasting protection powdery mildew on wheat (Görlach *et al.* 1996) but were later shown to have potential in many other plant pathogen systems.

Downy mildew of grapevines is a disease that may require frequent fungicide applications to prevent significant crop losses when conditions favour infection (warm wet weather) (Schwinn, 1981). Some of the most commonly used fungicides for downy mildew control have detrimental effects in the environment, are prone to pathogen resistance developing or their use is restricted by wineries due to residue concerns. For these reasons, investigating downy mildew control strategies using induced resistance is an attractive prospect.

Researchers in Israel and France have shown control of grapevine downy mildew via induced resistance in experimental systems (Cohen *et al*. 1999, Aziz *et al.* 2003) and in the field (Reuveni *et al.* 2001). There are also a number of products currently available on the Australian market which make general claims about boosting plant defences but are not registered as fungicides and do not have trial data to support their claims. The aim of this project was to screen a range of potential elicitors of plant defence identified from the literature and products in the marketplace against grape downy mildew and to evaluate the most promising candidates as alternatives to current fungicides.

4 Screening experiments

4.1 Introduction

A bioassay protocol for screening potential elicitors of plant defence against downy mildew was developed. Small potted grapevine cuttings were used to allow high throughput screening. The downy mildew inoculum concentration was optimised so that responses could be measured without defoliation of the vine which may occur at very high infection levels. Most products tested were sprayed onto foliage. *Trichoderma* biocontrol agents were added to potting mix as a drench.

4.2 Materials and methods:

4.2.1 Plant material

Grapevine rootlings (Chardonnay clone I10V1, Orlando Wyndham Nursery, Rowland Flat SA), or cuttings (Chardonnay clone I10V3, Cabernet Sauvignon clone G9V3,Victorian and Murray Valley Vine Improvement Association, Mildura, Vic) were stored at 4 °C until needed. When required, rootlings were removed from the cool-room and placed into potting mix (BJH/9321 reverse fines, Propine, Kilsyth, Vic) in 30 cm diameter pots. Cuttings were pruned to leave 2 nodes remaining and were potted in 8 cm diameter pots in the same potting mix. Plants were grown for 4-5 weeks in the glasshouse (20-28º C) prior to being used in experiments.

4.2.2 Screening trials 1 – 6

Elicitor application

Before spraying, the youngest expanded leaf with a length of at least 3 cm was tagged on the petiole with a piece of masking tape. The test solutions were applied to both upper and lower leaf surfaces until the point of run-off using a handheld atomiser. The potential inducers screened for activity against downy mildew are shown in table 4.1. In trials 1 and 2, each treatment was applied to 3 replicate plants. In trials 3-6, each treatment was applied to 5 replicate plants. Pots were arranged in a randomised block design in the glasshouse.

Table 4.1 Potential inducers and standard fungicides screened for activity against downy mildew in trials 1 - 6

Treatment	Active ingredient	Concentration of active ingredient or product used	Supplier	Trial(s)
Control	dH_2O	NA	NA	1,2,3,4,5,6
Brotomax®	Various	0.3 - 1 % (v/v)	Agrométodos (Spain)	1,2,3,5
$AlCl_3.6H_2O$	$AlCl_3$	10 - 45 mM	Sigma (NSW)	1,4
Bion® 50 WG	acibenzolar-S-methyl	0.7 - 1 mM	Syngenta (NSW)	1,3
BABA	β - aminobutyric acid	2 g/L	Sigma (NSW)	1,3
Methyl jasmonate	Methyl jasmonate	50 μM	Sigma (NSW)	3
Salicylic acid	Salicylic acid	1 g/L	Sigma (NSW)	3
Saccharin	Saccharin	3 mM	Sigma (NSW)	3
Arcadian®	Seaweed extract	750 mg/L	Organic Crop Protectants (NSW)	4
Chitosan (technical grade)	Chitosan	1 mg/mL	Mallinckrodt (Vic)	4,6
Laminarin	Laminarin	1 g/L	Sigma (NSW)	5
Vegemite	Yeast extract	1 g/L	Kraft foods (Vic)	5
Copper oxychloride 500	Copper oxychloride	1.5 g/L	Chemspray (Qld)	5
Aminogro®	Chitosan/amino acids	10 mL/L	Organic Crop Protectants (NSW)	6
Ecocarb®	Potassium bicarbonate	4 g/L	Organic Crop Protectants (NSW)	6
Maxicrop®	Seaweed concentrate	4 mL/L	Multicrop (Vic)	6

A stock solution of chitosan was prepared by dissolving 10 g/L chitosan (LL grade, Mallinckrodt, Vic) in 0.25 M HCl with constant stirring and then adjusting to pH 5.7 with NaOH. Chitosan was diluted to 1 g/L with distilled water prior to spraying. A saccharin solution was made up from a 300 mM saccharin (Sigma, NSW) stock solution was made up in acetone. The stock solution was then diluted 1 in 100 with distilled water to give a final saccharin concentration of 3 mM in 1% acetone. The saccharin solution was applied as a soil drench (40 mL per pot). A 1% acetone solution in distilled water was used as a control for the saccharin treatment.

Inoculation and disease assessment
An isolate of downy mildew (*Plasmopara viticola*) obtained from a vineyard in Knoxfield, Vic was maintained on Chardonnay vines in the glasshouse. Three days after application of elicitor solutions to foliage, test plants were inoculated with a freshly prepared zoospore suspension. Sporangia were washed from infected leaves and adjusted to the desired concentration. Different sporangial concentrations (2×10^4, 2×10^3 and 2×10^2 sporangia/mL) were tested in trial 2 to find the optimum for use in screening experiments. In later experiments 2×10^3 sporangia/mL was used (Trials 3-6). Leaves were inoculated by spraying the underside of all leaves on the shoot with a hand-held atomiser. Inoculated shoots were covered with polyethylene bags which were sealed around the stem to maintain leaf wetness overnight. The polyethylene bags were removed the following morning.

After 12-14 days incubation in the glasshouse (20-28 º C) shoots were covered with plastic bags overnight to allow sporulation to occur. The following morning the leaves were detached from the shoot and the underside of leaves were photographed with a digital camera (Olympus E-20P, Olympus, Japan). The area of the leaf covered with sporulation downy mildew and the total leaf area was measured using image analysis software (Sigmascan Pro, Jandel Scientific USA) for each leaf position on the shoot. The notation used for leaf position was 0=youngest leaf at time of spraying ($\geq$ 3 cm in length), -1 = adjacent younger leaf, +1=adjacent older leaf etc (Figure 1). A measure of the diseased area per shoot was calculated from the infected leaf area and total leaf area from the most susceptible leaf positions on the shoot, generally leaf positions –1 through to +2 or +3 but this was dependant on growth rate of the plants during the experiment.

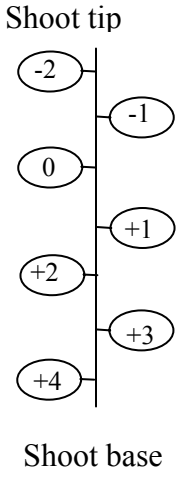


Figure 4.1 Leaf position notation used for trials 1 – 5. The position is relative to the youngest expanded leaf (≥3 cm length) at the time of elicitor application (leaf 0).

Sporangia were then washed from leaves with a known volume of water containing 2 drops/L Tween 80 and counted using a haemocytometer. Leaf areas were measured by image analysis and the number of sporangia produced per cm^2 of leaf was calculated.

4.2.3 Screening Trial 7, *Trichoderma*

Elicitor application

Potted vines were grown for 1 month as previously described (section 4.1.1) before soil inoculation with *Trichoderma* formulations. Two formulations were tested, Trich-A-Soil® (Organic Crop Protectants, NSW) containing *Trichoderma harzianum* strains BC702 and BC703 and Trichoflow® Ali 52 containing powdered *Trichoderma atroviride* (experimental formulation, Agrimm, NZ). Both formulations were dispersed in distilled water and 50 mL applied to the potting mix. Trich-A-Soil® was applied to soil at a rate of 14 g/L and Trichoflow® was applied at 10 g/L. The control treatment had 50 mL of dH_2O applied to the potting mix. Each treatment was applied to nine replicate plants.

Inoculation and disease assessment

Plants were inoculated with downy mildew (2×10^3 sporangia/mL) 17 days after *Trichoderma* application using the method described in section 4.1.2. Diseased leaf area and sporulation was assessed after 14 days incubation. Different leaf position notation to that used in trials 1 –5 was used in trial 6. Leaf position was recorded at the time of disease assessment, with the leaf closest to the shoot tip being leaf 1 and with the leaf number increasing down the shoot. The average diseased leaf area was calculated for young leaves (leaves 1-3) and older leaves (leaves 4-6). Sporangia were washed from all 6 leaves (young and older leaves) and counted using a haemocytometer. Leaf areas were measured by image analysis to determine sporangia production per cm^2 of leaf area.

To test if the *Trichoderma* had survived and colonised the potting mix, dilution plates were made from the potting mix after disease assessment. Approximately 10 grams of soil was added to 90 mL of distilled water in a plastic bag and mixed in a bag mixer (Bagmixer 400, Interscience, France) for 30 sec. Serial dilutions of 10^{-1}, 10^{-2} and 10^{-3} were made and 0.5 mL of soil solutions were spread across the surface of TSA (*Trichoderma* selective agar, developed by Lincoln University, NZ) plates. TSA plates were incubated at room temperature for 2 weeks and then *Trichoderma* colonies were counted.

4.2.4 Data analysis

Data were transformed where appropriate and analysed by ANOVA (Gentstat 7, VSN International, UK). Back transformed mean values are presented.

4.3 Results and discussion:

4.3.1 Initial screening

Trial 1:

$AlCl_3$ and Brotomax® were the only products to give significant disease control in terms of both leaf area infected and sporangia produced per cm^2 of leaf area (Table 4.2) when plants were inoculated with a high inoculum dose (2×10^4 sporangia/mL). Bion® and BABA both resulted in reduced sporulation but this was not statistically significant. $AlCl_3$ caused considerable phytotoxicity to leaves while Brotomax treatment left small dark spots on leaves (Figure 4.2).

Table 4.2 Effect of potential elicitors of plant defence on downy mildew colonisation on Chardonnay grapevine leaves inoculated with 2×10^4 sporangia/mL. *n*=3

Treatment	Mean diseased leaf area per shoot (%)	Sporangia production/cm^2 (x 10^3)
Control	12.4 a	17.4 a
BABA	13.2 a	10.2 a
Bion®	12.7 a	11.7 a
1% Brotomax®	0.5 b	0.6 b
45 mM $AlCl_3$	0.0 b	0.0 b

Values within a column followed by the same letter are not significantly different at the 5% level.



(a) (b)

Figure 4.2 Phytotoxicity symptoms on Chardonnay leaf treated with 45 mM $AlCl_3$ (a) and dark spots on leaves following treatment with 1 % (v/v) Brotomax® (b).

4.3.2 The effect of inoculum concentration

Trial 2:

Different inoculum concentrations were trialed to optimise the screening bioassay using only Brotomax® and control (dH_2O) treatments (Table 4.3). The disease reduction relative to the control treatment when Brotomax was applied was very similar at inoculum doses of 2 x 10^2 and 2 x 10^3 but was reduced at 2 x 10^4 sporangia/mL. The sporangia concentration of 2 x 10^3 was chosen for all subsequent experiments.

Table 4.3 The effect of inoculum concentration on disease reduction relative to the control treatment (dH_2O) when 1 % Brotomax® was applied. *n*=3.

Applied inoculum concentration (Sporangia/mL)	Reduction in diseased leaf area per shoot (%) after application of 1% (v/v) Brotomax®
2 x 10^2	97
2 x 10^3	95
2 x 10^4	86

Trial 3:

In this screening experiment, plants were inoculated with 2 x 10^3 sporangia/mL. Brotomax® resulted in 100% disease control (Table 4.4). Bion® was the only other product to give a substantial reduction in disease (approximately 60%) which was also statistically significant. This result is in contrast to the first screening trial where no reduction in downy mildew occurred when 0.7 mM Bion® was applied.

Table 4.4 Average downy mildew severity on leaves (positions 0 to +2) of Chardonnay grapevines treated with elicitors. *n*=5

Treatment	Mean diseased leaf area per shoot (%)
Control	20.67 a
1 % (v/v) Acetone*	25.38 a
3 mM Saccharin	15.22 ab
1 mM Bion®	10.29 b
1% Brotomax®	0.00 c
50 µM Methyl Jasmonate	23.46 a
2 g/L BABA	16.03 ab
1 g/L Salicylic acid	25.90 a

* 1% acetone was used as the control treatment for saccharin which was dissolved in 1 % acetone

Values within a column followed by the same letter are not significantly different at the 5% level.

Trial 4:

The application of Acadian® to vine foliage resulted in significantly higher disease than the control treatment while chitosan and $AlCl_3$ treatments had significantly less disease than the control (Table 4.5Table 4.5).

Table 4.5 Downy mildew severity on leaves of Chardonnay grapevines treated with inducers. *n*=5.

Treatment	Mean diseased leaf area per shoot (%)
Control	25.06 a
1 g/L chitosan	0.00 b
0.75 g/L Arcadian®	42.56 c
10 mM $AlCl_3$	0.02 b

Values within a column followed by the same letter are not significantly different at the 5% level.

Trial 5:

The application of yeast extract and laminarin to foliage did not reduce the severity of downy mildew (Table 4.6). Only the standard copper fungicide and 0.3% Brotomax reduced disease significantly by 100% and approximately 90% respectively.

Table 4.6 Downy mildew severity on leaves of Chardonnay grapevines treated with inducers. *n*=5

Treatment	Mean diseased leaf area per shoot (%)
Control	4.16 a
0.3 % Brotomax®	0.49 b
1 g/L Laminarin	4.07 a
1 g/L Yeast extract	3.93 a
3 g/L Copper	0.00 b

Values within a column followed by the same letter are not significantly different at the 5% level.

Trial 6:
Complete disease control was achieved with the technical grade chitosan treatment while the other chitosan containing product, Aminogro® did not have a significant effect on downy mildew severity (Table 4.7). The technical grade chitosan produced phytotoxicity symptoms one day after application in this trial. No such phytotoxicity symptoms had been observed in previous experiments. Ecocarb® reduced disease by 99% (significant, $P<0.05$). Maxicrop® had higher disease severity that the control but this was not statistically significant ($P>0.05$).

Table 4.7 Downy mildew severity on leaves of Chardonnay grapevines treated with inducers. *n*=5

Treatment	Mean diseased leaf area per shoot (%)
Control	10.7 a
Ecocarb®	0.1 b
Aminogro®	9.2 a
Chitosan (technical grade)	0.0 b
Maxicrop®	14.2 a

Values within a column followed by the same letter are not significantly different at the 5% level.

4.3.3 Trichoderma as an inducer of resistance against downy mildew

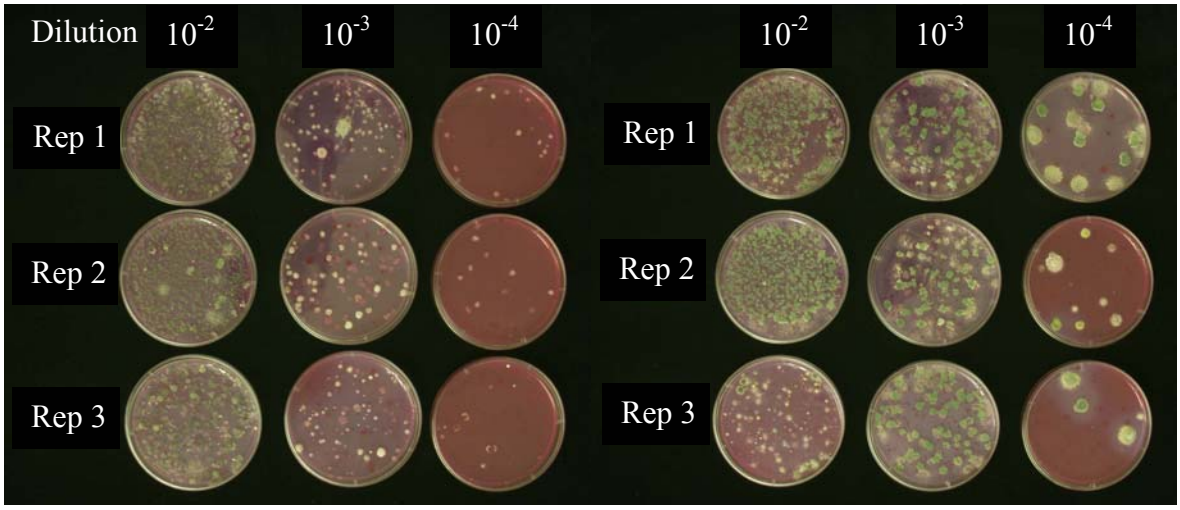
Trial 7:
No significant decrease in the amount of leaf area infected with downy mildew was observed on plants grown in soil treated with either *Trichoderma* formulation (Table 4.8). There were also no significant reductions in sporulation of the pathogen for either *Trichoderma* treatment although there was a significant difference in sporulation between the two *Trichodermas* (Table 4.8). Dilution plates made from the potting mix after disease assessments were completed showed that both *Trichodermas* were able to survive and colonise the potting mix (Figure 4.3). CFU (colony forming unit) counts for Trich-A-

Soil® and Trichoflow® were 2.2 ±0.3 x 10^5 and 1.0 ±0.2 x 10^5 per gram of potting mix respectively at the end of the experiment.

Table 4.8 Leaf area infected with downy mildew on plants treated with different Trichoderma formulations. Sporangia produced per cm^2 of leaf area was calculated for leaf positions 1-6. *n*=9.

Treatment	Young leaves (positions1-3)	Old leaves (positions 4-6)	Sporangia/cm^2 (x 10^3) (positions 1-6)
Control	24.6 a	4.17 a	35.2 ab
Trich-A-Soil®	17.4 a	2.03 a	23.6 a
Trichoflo®	27.4 a	4.48 a	50.6 b

Values within a column followed by the same letter are not significantly different at the 5% level.



(a) (b)

Figure 4.3 Dilution plates of *Trichoderma* treated soil (a) Trich-A-Soil® treated soil, (b) Trichoflow Ali 52® treated soil. *Trichoderma* colonies are blue/green in appearance.

4.4 Conclusions

The efficacy of inducers against downy mildew in glasshouse pot trials is summarised in Table 4.9.

Table 4.9 Summary of inducer activity against downy mildew in glasshouse pot trials

Inducer	Concentration	Disease reduction relative to control treatment (%)	Significance
Brotomax®	1 % (v/v)	96-100	*
Brotomax®	0.3% (v/v)	88	*
$AlCl_3$	45 mM	100	*
$AlCl_3$	10 mM	99.9	*
BABA	2 g/L	0	
Bion®	0.7-1 mM	0 - 60	*
Saccharin	3 mM	44	
Methyl Jasmonate	50 μM	0	
Salicylic acid	1 g/L	0	
Laminarin	1 g/L	2.2	
Yeast extract	1 g/L	5.5	
Arcadian®	750 mg/L	0	
Chitosan	1 mg/mL	100	*
Trichoflow®	10 g/L	0	
Trich-A-Soil®	14 g/L	20	
Aminogro®	10 mL/L	14	
Maxicrop®	4 mL/L	0	
Ecocarb®	4 g/L	99	*

* significant ($P<0.05$)

Brotomax®, $AlCl_3$, chitosan and potassium bicarbonate (Ecocarb®) all provided high levels of control of downy mildew. Aminogro®, a product that contains chitin from processed crustacean waste (and has been considered to be a chitosan containing formulation by other researchers (Walker *et al* 2004), was not effective against downy mildew. Brotomax® is a nutrient formulation with claimed plant defence boosting capabilities. Another nutrient formulation (Maxicrop®) which contained less copper than Brotomax®, was also tested but had no activity against downy mildew. Thus, anti-downy mildew activity of Brotomax® is not common to all nutrient formulations and may be due to its copper content or other unknown components. Only a single experiment was conducted with the potassium bicarbonate treatment (Ecocarb®) after learning of anecdotal evidence of activity against downy mildew (D. Madge *pers com*). Ecocarb® was highly effective in this one trial but did not give 100% disease control while the chitosan treatment in this experiment did.

$AlCl_3$ caused considerable phytotoxicity at doses that gave high levels of disease control and at higher rates resulted in defoliation of plants. At 1 mM no phytotoxicity was observed with $AlCl_3$ but disease control was less than 90%. Excess aluminium in soils can be toxic to grapevines and therefore $AlCl_3$ would not be a good candidate for a crop protection chemical. There was some evidence of disease control with Bion®, with a 60% disease reduction (non-significant) in one trial, although there was no disease reduction in another trial. This finding is in agreement with Italian research which has shown Bion® can reduce downy mildew on grapevines but high rates are required resulting in phytotoxicity problems and it does not consistently provide a level of control that would be acceptable in commercial viticulture (Pertot *pers com*).

Soil application of *Trichoderma* biocontrol agents have been reported to give disease reduction on foliage through induction of host defence responses in some plant/pathogen systems. Induced resistance is involved in the suppression of Botrytis grey mould symptoms by the biocontrol agent *Trichoderma harzianum* T39 in tomato, lettuce, pepper, bean and tobacco (De Meyer *et al.* 1998) and application of *Trichoderma asperellum* (T-203) induces defence responses in cucumber again *Pseudomonas syringae* (Yedidia *et al.* 2003). In contrast, no significant control of grapevine downy mildew was observed when two different formulations of *Trichoderma* were added to the potting mix in this research.

β-aminobutyric acid (BABA) and laminarin (a β1,3-glucan extracted from seaweed), have also been reported to elicit defence responses against downy mildew on grapevines (Cohen *et al.* 1999, Reuveni *et al.* 2001, Aziz *et al.* 2003). However, they were not found to be effective in this present study.

Brotomax® and chitosan gave levels of disease control similar to the standard fungicides in these glasshouse trials and so warranted further testing. One formulation of chitosan (technical grade) caused phytotoxicity in one trial but not others. A single experiment with Ecocarb® (potassium bicarbonate) also showed very promising results against downy mildew. Although complete control was not achieved with Ecocarb®, disease was reduced by 99%. This non-toxic, food grade product has APVMA registration for powdery mildew control in grapes and its price is comparable to currently used fungicides. Due to time constraints, Ecocarb was not tested in the field in this project but is worth investigating further after these initial promising results.

5 Characterising elicitor activity

5.1 Introduction

In order to optimise disease control with elicitor treatments, dose-response experiments and different spray timings were trialed. Downy mildew attacks developing bunches as well as leaves so efficacy for protecting young bunch tissue from downy mildew attack at flowering was also tested. Often the best results with elicitors of plant defence are obtained in combination with low doses of fungicides or bactericides (Ruess *et al.* 1996, Tally *et al.* 1999). For this reason, Brotomax® and chitosan treatments were also tested in combination with low doses of the standard fungicide, copper oxychloride to test for synergistic effects.

5.2 Materials and Methods

5.2.1 Brotomax® dose-response

Brotomax® solutions at concentrations of 1 %, 0.5%, 0.3%, 0.1% and 0% (control) were applied to the leaves of potted Chardonnay vines (grown as previously described) to the point of run-off. Three days after chemical application, plants were inoculated with downy mildew, incubated and assessed as previously described.

5.2.2 Chitosan dose-response

Chitosan solutions at concentrations of 1, 0.5, 0.25, 0.124 and 0 (control) g/L were prepared from a chitosan liquid formulation (Ellis and Associates, Melbourne) and were applied to the leaves of potted Chardonnay vines to the point of run-off. Technical grade chitosan (LL grade, Mallinckrodt Vic) was applied at 1 g/L as a comparison. Three days after chitosan application, plants were inoculated with downy mildew, incubated and assessed as previously described.

5.2.3 Post-inoculation activity against downy mildew

Potted Chardonnay grapevines were inoculated with downy mildew as previously described. One day after inoculation, plants were sprayed to run-off with solutions containing 1 mg/mL chitosan (LL grade, Mallinckrodt Vic), 1.5 g/L copper (Copper oxychloride, 500 g/Kg Cu, Chemspray, NSW), 5 mL/L Agri-fos Supa 400 (400 g/L Phosphorous acid, Agrichem, Qld). In a separate experiment, 1 % (v/v) Brotomax® was applied both 2 days prior to inoculation and 1 day post-inoculation. Five replicate plants were sprayed with each treatment in both trials. Plants were incubated in the glasshouse and assessed for disease as previously described.

5.2.4 Synergy of inducer treatments with fungicides

Low doses of copper oxychloride were applied in combination with low doses of Brotomax® and chitosan treatments to potted Chardonnay grapevines to test for

synergistic effects. In the first experiment, the treatments applied were Brotomax at 0.1 % (v/v) alone, 0.1 g/L copper (copper oxychloride) alone, Brotomax at 0.1 % (v/v) and 0.1 g/L copper (copper oxychloride) in combination and control (dH_2O). In the second experiment the treatments were 0.1 g/L chitosan (LL technical grade) alone, 0.5 g/L copper (Copper oxychloride 500) alone, 0.1 g/L chitosan (LL technical grade) and 0.5 g/L copper (Copper oxychloride 500) combined and control (dH_2O). In both experiments five replicate plants were sprayed to the point of run-off. Plants were inoculated with downy mildew (2×10^3 sporangia/mL three days after spraying) and disease was assessed after 14 days as previously described (section 4).

5.2.5 Activity on flower tissue against downy mildew

Elicitor activity against downy mildew on flower tissue was tested on 6 year-old Chardonnay vines grown in pots in the glasshouse which had between 3 and 11 inflorescences per plant. The plants were treated with the following elicitor/fungicide solutions at 80 % cap-fall; 1 % (v/v) Brotomax®, 3 g/L copper oxychloride, 1 mg/mL chitosan, control (dH_2O) 3 days prior to inoculation with 1×10^4 sporangia/mL using the protocol previous described. Phosphorous acid (Agri-fos Supa 400, 400 g/L Phosphorous acid, Agrichem, NSW) was applied at the rate of 1.2 mL/L (a.i.) 2 days post-inoculation. Half of the inflorescences on each plant were covered with polyethylene bags during spraying to prevent any deposition of the test product while the other half of the inflorescences were sprayed along with all the foliage. Five replicate plants were treated with each elicitor/fungicide. Plants were arranged in the glasshouse in a complete randomised block design and incubated for 10 days. Sporulation was induced by covering inflorescences with plastic bags overnight. The following morning, the percentage of flowers infected per inflorescence was recorded for each plant using a dissector microscope. The mean percentage of flowers infected per inflorescence was calculated separately for flowers sprayed directly and those covered with plastic bags during elicitor/fungicide application.

5.3 Results and discussion

5.3.1 Brotomax® dose-response relationship

Disease control increased with increasing Brotomax® dose and all doses tested gave a significant disease reduction relative to the control (Figure 5.1). The highest level of disease control occurred at 1 % (v/v) Brotomax® although this wasn't significantly different to the standard fungicide, copper oxychloride, treatment at either 0.2 or 1.5 g/L Cu or Brotomax® at 0.5 and 0.3 % (v/v). The lower concentration of the standard fungicide (0.2 g/L Cu) was calculated to have the same copper concentration as 1 % (v/v) Brotomax® solution based on the manufacturer's analysis. While not significantly different, the lower rate of copper oxychloride treatment did have more disease than 1% Brotomax® treatment suggesting ingredients other than copper in Brotomax® may contribute to its activity against downy mildew.

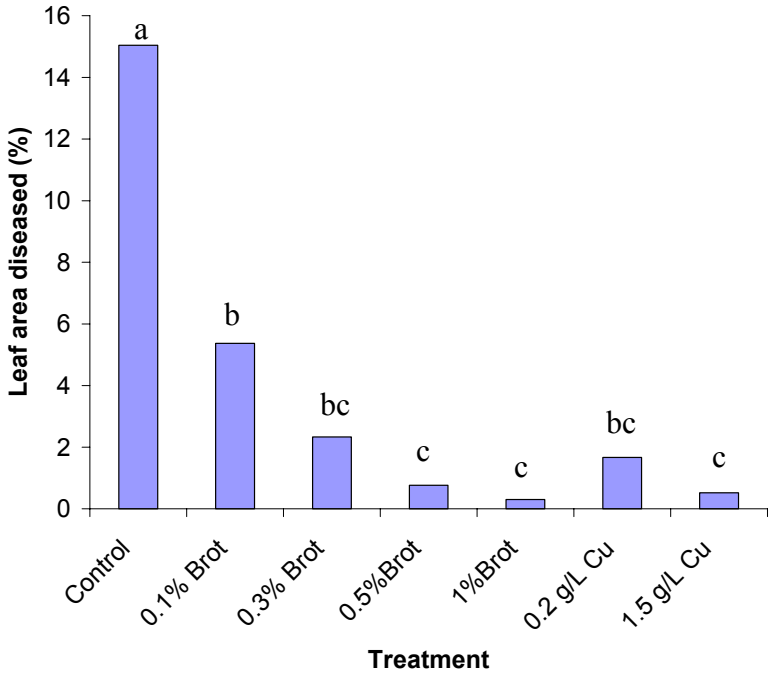


Figure 5.1 Brotomax® (Brot) efficacy against downy mildew at 0.1, 0.3, 0.5 and 1% (v/v). A standard fungicide treatment, copper oxychloride (Cu) was also tested at 0.2 and 1.5 g/L (a.i). *n*=5. Values with the same letter above the bar are not significantly different at the 5% level.

5.3.2 Chitosan dose-response

All chitosan concentrations tested reduced disease by 90% or more (Figure 5.2). There was no significant difference in disease control for liquid chitosan concentration between 0.125 and 0.5 g/L but disease control was significantly higher when 1 g/L chitosan was applied.. The liquid formulation of chitosan had higher efficacy at 1 g/L than the technical grade but this difference was not statistically significant (Figure 5.2).

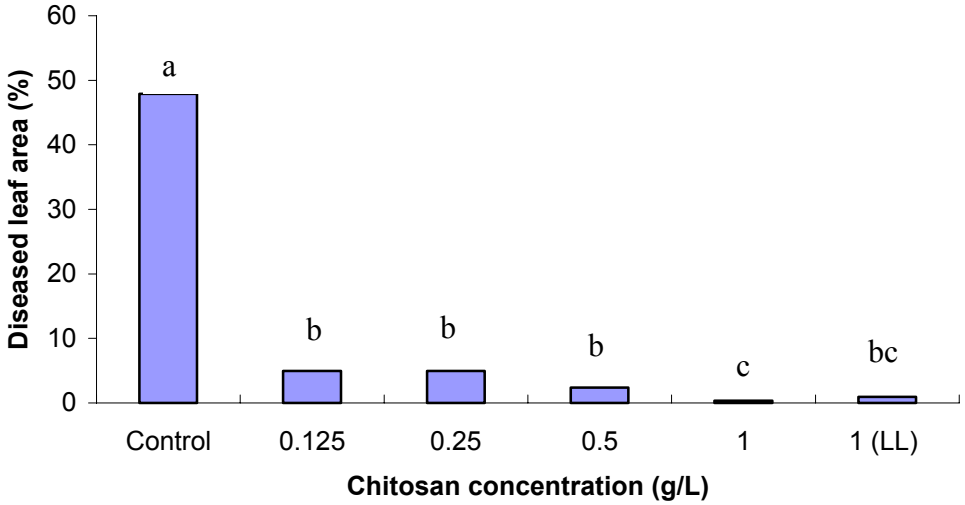


Figure 5.2 Chitosan dose-response for the chitosan liquid formulation against downy mildew. Technical grade chitosan (LL) was used as a comparison at 1 g/L. Values with the same letter above the bar are not significantly different at the 5% level.

5.3.3 Timing of elicitor application

There was no significant post-infection activity for chitosan, $AlCl_3$ or copper oxychloride when applied one day post-inoculation (Table 5.1). Phosphorous acid showed strong post-infection activity against downy mildew.

Table 5.1 Activity of inducers against downy mildew on grapevine foliage when applied one day post-inoculation. *n*=5.

Treatment	Mean diseased leaf area per shoot (%) (leaf positions -1 to +2)
Control	53.3 a
Chitosan	40.0 a
Copper oxychloride	51.6 a
Phosphorous acid	0.04 b
$AlCl_3$	35.4 a

Values within a column followed by the same letter are not significantly different at the 5% level.

Brotomax application only resulted in significant disease reduction relative to the unsprayed control when applied pre-inoculation (Table 5.2).

Table 5.2 Activity of 1 % (v/v) Brotomax® against downy mildew on leaves of Chardonnay vines at different spray timings. *n*=5.

Spray timing	Mean diseased leaf area per shoot (%) (leaf positions -1 to +2)
Control (unsprayed)	27.5 a
2 days pre-inoculation	0.56 b
1 day post-inoculation	19.5 a

Values within a column followed by the same letter are not significantly different at the 5% level.

5.3.4 Synergy between inducers and copper oxychloride

There was no significant increase in disease control when Brotomax® was applied in combination with a low dose of copper oxychloride (Table 5.3). The low dose of Brotomax® (0.1% v/v) did provide a significant reduction in disease severity (approximately 66%) but this level of disease control would not be acceptable in a commercial vineyard.

Table 5.3 Efficacy of low doses of Brotomax®, copper oxychloride (Cu) and both treatments combined for downy mildew control on leaves. *n*=6

Treatment	Mean diseased leaf area per shoot (%) (leaf position –1 to 3)
Control	14.90 a
0.1 % Brotomax®	5.29 b
0.1 % Brotomax ® + 0.1 g/L Cu	0.21 c
0.1 g/L Cu	0.40 c

Values within a column followed by the same letter are not significantly different at the 5% level.

There was no evidence of synergy between chitosan and copper at reduced dose rates (Table 5.4). In fact, the combination treatment was significantly less effective than chitosan alone suggesting antagonism between copper and chitosan

Table 5.4 Efficacy of low doses of chitosan, copper oxychloride (Cu) and both treatments combined against downy mildew on leaves. *n*=6

Treatment	Mean diseased leaf area per shoot (%) (leaf positions –1 to 3)
Control	38.75 a
0.1 g/L Chitosan	4.47 b
0.1 g/L Chitosan + 0.05 g/L Cu	14.03 c
0.05 g/L Cu oxychloride	17.33 c
LSD (P=0.05)	7.7

Values within a column followed by the same letter are not significantly different at the 5% level.

5.3.5 Antifungal activity on flower tissue

Both chitosan and Brotomax® reduced downy mildew severity on inflorescences and were not significantly different to the standard fungicides, copper oxychloride and phosphorous acid when the inflorescences were directly sprayed (Table 5.5).

Table 5.5 Efficacy of elicitors and fungicides for control of downy mildew on inflorescences when entire plant sprayed (contact activity) or when only leaves were treated (systemic activity). *n*=5

Treatment	Flowers infected per inflorescence (%)	
	Contact activity	Systemic activity
Control	45.99 a	46.34 a
1% Brotomax	2.29 b	27.15 a
1 g/L Chitosan	1.28 b	34.72 a
3 mL/L Phosphorous acid	2.29 b	5.45 b
1.5 g/L Cu (oxychloride)	0.06 b	33.89 a

Values within a column followed by the same letter are not significantly different at the 5% level.

There was no significant reduction in downy mildew severity on flowers when only the foliage was sprayed for any products except phosphorous acid which is known to be a highly systemic fungicide (Cohen and Coffey 1986).

5.4 Conclusions

Both Brotomax® and chitosan gave high levels of downy mildew control on leaf and flower tissue. However, neither product showed significant post-infection activity when tested on previously inoculated leaves. Thus, both Brotomax® and chitosan must be applied before downy mildew infection has occurred for them to be effective in

controlling the disease. This is consistent with what would be expected of a defence-inducing compound. There was no evidence of synergy between low doses of either Brotomax® or chitosan when applied in combination with low doses of copper oxychloride. There was also no evidence of systemic protection of flower tissue when Brotomax® and chitosan were only applied to foliage. This suggests that only sprayed flower tissue is protected from disease and therefore achieving good spray coverage of the target will be important to achieve a high level of disease control.

6 Mode of action of elicitors

6.1 Introduction

Experiments were conducted to determine the mode of action of potential elicitors of plant defence against downy mildew. Inducers of resistance may or may not have direct toxicity to pathogens and may be able to protect tissues distant to the site of application in the case of systemic acquired resistance (SAR). Several pathogenesis-related proteins (PR-proteins) are known to accumulate during SAR (Kunkel and Brooks 2002). Some of these PR-proteins are hydrolytic enzymes including chitinases and β 1,3-glucanases and the increased activities of these enzymes can be a marker of SAR.

6.2 Materials and methods

6.2.1 Activity against zoospores

Zoospore suspensions were prepared as previously described (section 4.1.3). Stock solutions of Brotomax® (1% v/v), $AlCl_3$ (45 mM) and chitosan (10 g/L of LL technical grade) were prepared as previously described (section 4.1.2) in distilled water. To test for their effects on the release of zoospores from sporangia, 100 µL of test solution were added to 900 µL of freshly prepared sporangia suspension in a 1 mL tube. The number of empty sporangia was recorded after 1 hour. To test for activity against already released zoospores, freshly prepared sporangia suspensions were left at room temperature for 45 minutes to allow zoospore release before test products were added. Test solutions were diluted 1 in 10 with zoospore suspension on a concave microscope slide and mixed. Distilled water was added to the control. The activity of zoospores was observed for 3 minutes using a compound microscope (200 X) to see if the zoospores remained motile.

6.2.2 Systemic and translaminar activity

To test for systemic activity, only leaves at the base of the shoot were sprayed with elicitor solutions. A polyethylene bag was placed over the shoot below the youngest open leaf (with a length of at least 3 cm) to prevent spray being deposited on this leaf and all younger leaves (Figure 6.1).

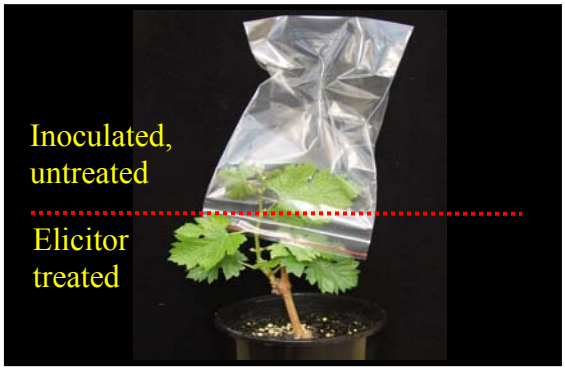


Figure 6.1 Covering of younger leaves during elicitor application

The bag was removed immediately after spraying. The whole shoot was inoculated 3 days after sprays were applied as previously described in section 4.1.3.

Translaminar activity was measured by spraying only the upper surface of leaves with test solutions. Three days after spraying, the leaves were inoculated on the underside as previously described in section 4.1.3.

6.2.3 PR-protein hydrolytic enzyme activities

Elicitors were applied to foliage of potted grapevines (grown as previously described) by spraying with either distilled water (control) or 1 % (v/v) Brotomax® or by abrading the upper leaf surface with sandpaper and adding two 20 µL drops of 7.2 mM salicylic acid with a micropipette. Three replicate plants were treated with each inducer treatment.

Chitinase and β 1,3-glucanse activity in leaves was determined 3 days post elicitor application. The youngest expanded leaf (≥3 cm length) at the time of elicitor application was removed and ground to a powder in a mortar and pestle in liquid nitrogen. Approximately 0.15 g of ground leaf tissue was added to 1 mL of cold 0.05 M sodium acetate buffer (pH 5) containing 5 mg/mL polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at 14,000 g for 20 min at 4° C. The chitinase activity of the supernatant was determined by measuring the liberation of dye containing products from the dye-labelled soluble chitin substrate carboxymethyl-chitin-remazol brilliant violet 5R (Loewe Biochemica, Germany) using the protocol of Wirth and Wolf (1990). The 1,3-glucanase activity was determined using the laminarin dinitrosalicylate method of Abeles *et al*. (1971) as modified by Reuveni (1998).

6.3 Results and discussion

6.3.1 Activity against zoospores

$AlCl_3$, Brotomax® and chitosan prevented the release of zoospores from sporangia completely at concentrations of 4.5mM, 0.1% and 0.1 g/L respectively. $AlCl_3$, Brotomax® and chitosan also appeared to be highly toxic to zoospores as motility ceased within 1 minute of the addition of the test chemical. Chitosan has been reported to have antifungal activity against other plant pathogenic fungi *in vitro* (Benhamou 1992, Bell *et al*. 1998)

6.3.2 Systemic and translaminar activity

Disease on upper leaves, measured as both leaf area infected and pathogen sporulation, was reduced when elicitors were only applied to the lower leaves of the plants. Both $AlCl_3$ and Brotomax® gave similar levels of systemic protection against downy mildew on 2 year old Chardonnay vines (Table 6.1).

Table 6.1. Disease reductions on unsprayed upper leaves of plants that had elicitors applied to lower leaves. Area infected data was angular transformed and sporangia/cm^2 data was square root transformed prior to ANOVA. *n*=5

Treatment	Diseased area reduction (%)	Sporulation reduction (%)	Test plants
$AlCl_3$	54 s	61 s	Chardonnay (2 years old)
Brotomax	51 n.s	56 n.s	Chardonnay (2 years old)
Brotomax	65 s	71 n.s	Cabernet Sauvignon (cuttings)
Chitosan	-2 n.s	NA	Chardonnay (cuttings)

s – significant at 5% level
n.s. – not significant at 5% level
NA – not assessed

This effect was only statistically significant for $AlCl_3$ (Table 6.1). However, on Cabernet Sauvignon plants grown from cuttings, diseased leaf area on upper leaves was significantly lower than the control when the lower leaves were sprayed with 1% Brotomax® (Table 6.1). The reduction in sporulation was however not significant (Table 6.1)

When measuring translaminar activity (only the upper surface of all leaves were sprayed), Both Brotomax® and $AlCl_3$ decreased leaf area infected and sporulation relative to the control. However, this effect was only statically significant ($P<0.05$) for the leaf area infected for Brotomax® (Table 6.2).

Table 6.2 Translaminar activity of inducers of resistance against downy mildew on Chardonnay grapevine leaves. *n*=5

Treatment	Inducer applied to	Leaf area infected (%)	Sporangia produced/cm^2 (x 10^3)
Control		19.4 a	40.4 a
$AlCl_3$	Upper surface	9.2 a	23.4 a
Brotomax®	Upper surface	8.2 b	17.4 a

Values within a column followed by the same letter are not significantly different at the 5% level.

6.3.3 Pathogenesis related protein hydrolytic enzyme activity assays

No increases in chitinase or β 1,3-glucanase activity relative to the control were detected for either Brotomax® or salicylic acid + wounding treatments when tissue homogenates were prepared 3 days after elicitor application from the youngest expanded leaf (>3cm length) at the time of elicitor application (Table 6.3).

Table 6.3. PR-protein hydrolytic enzyme activities 3 days post inducer application. Means were calculated from 3 replicate leaf samples ± SEM.

Treatment	Chitinase activity (increase in A540nm/g tissue)	β 1,3-glucanase activity (mg/mL glucose equivalents/g tissue)
Control	0.54 ± 0.18	4.18 ± 0.25
Salicylic acid +wounding	0.58 ± 0.13	4.12 ± 0.11
Brotomax®	0.43 ± 0.10	4.37 ± 0.16

6.4 Conclusions

All products with high efficacy against downy mildew identified in the screening trials were toxic to zoospores. There was some systemic protection of unsprayed leaves on treated plants for Brotomax® and $AlCl_3$ although this effect was not always statistically significant. No systemic protection on unsprayed leaves was observed on chitosan treated plants. Efficacy against downy mildew was only observed when products were applied prior to inoculation. No reductions in disease severity occurred when products were applied post-inoculation. No markers of an induced resistance response such as increased pathogenesis related-protein hydrolytic enzyme activities (chitinase and β 1,3-glucanase) were observed.

7 Performance of elicitors in the field

7.1 Introduction

Products that showed high levels of efficacy against downy mildew in earlier glasshouse trials were tested under field conditions in a commercial vineyard. Both developing bunches and shoots were inoculated with downy mildew to ensure high disease pressure during the trial. The trial block was also inoculated with *Botrytis* to test for any side effect of the downy mildew treatments on this pathogen.

7.2 Materials and methods

7.2.1 Trial design

The elicitor treatments that showed promise in glasshouse testing, chitosan and Brotomax®, were tested under field conditions in a commercial vineyard in Wantirna, Vic. Chitosan was tested at 1 mg/mL and Brotomax® at 1% (v/v) and were compared to the standard fungicides copper oxychloride (Chemspray Qld) at 3 mg/mL and phosphorous acid (Agriphos 600) at 2.4 g/L (a.i). Distilled water was applied to a control treatment. Elicitors and the protectant fungicide (copper oxychloride) were applied at the 80% cap-fall growth stage, 3 days prior to inoculation. Phosphorous acid, a fungicide with post-infection activity, was applied one day post-inoculation. All sprays were applied with an electric backpack sprayer (Silvan, Vic) at the rate of 8 L/plot (10 m length). Each treatment was applied to four replicate plots of four vines in a randomised block design

7.2.2 Inoculation

Downy mildew

Downy mildew inoculum was prepared as previously described (Section 4.1.3) and adjusted to 4 x 10^4 sporangia/mL. Five shoots and five inflorescences per plot were marked with flagging tape (so inoculated tissues could be identified when assessments were due) and were sprayed to run-off with sporangial suspension. Plastic bags were placed over inoculated shoots and inflorescences to maintain wetness while spores infected. Inoculations were carried out in the early evening to prevent bagged tissues heating excessively in bright sunlight. The following morning plastic bags were removed. After nine days incubation, plastic bags were placed over inoculated tissues to induce sporulation. The following morning, shoots and inflorescences were removed and taken back to the lab for disease assessment.

Botrytis inoculation

The *Botrytis cinerea* inoculum was produced from isolate B33, obtained from Dr Tonya Wiechel of Monash University (Frankston, Vic). Cultures were grown for 2 weeks on PDA (potato dextrose agar) at 20^o C in the dark and sporulation was induced by placing cultures under a long wave UV light overnight. A spore suspension was prepared in distilled water containing 3 drops /mL of surfactant (Tween 20) to stop spores clumping

together. The spore concentration was adjusted to 1.75 x 10^5 spores per mL using a haemocytometer. Two days after applying elicitor and fungicide treatments, five inflorescences per plot were inoculated with the *Botrytis* spore suspension. Inoculations were completed between 5 pm and 8 pm. Inoculated inflorescences were bagged, using zip-lock plastic bags (15 cm × 23 cm), to maintain high humidity and allow the Botrytis spores to germinate. The inoculated inflorescences were tagged so they could be easily identified. The plastic bags were all removed by 9 am the following morning.

7.2.3 Disease assessment

Downy mildew

Downy mildew infection was assessed on the five inoculated inflorescences per plot as previously described (section 5.2.4). Downy mildew infection on shoots was calculated as the average percent leaf area infected for leaf positions 3 to 7. Percentage leaf area infected was measured using image analysis as previously described. The first two leaves on the shoot were not used in the calculation of shoot infection because these leaves were not expanded at the time of elicitor/fungicide treatment and inoculation.

Botrytis

Botrytis infection on the five inoculated inflorescences from each treatment plot was assessed by moist incubation in the laboratory. Inflorescences collected from the field were surface sterilised in 1% sodium hypochlorite for 3 min and washed twice with distilled water, then placed inside plastic takeaway food containers (G750, Genfac plastics, Melbourne) on to of a piece of paper towelling wetted with distilled water in order to maintain a humid environment. Inflorescences were placed in a freezer (-18^o C) overnight to terminate host resistance in the cells of the cuticular membrane without damaging host tissue (Coertze and Holz, 1999). The following morning, inflorescences were removed from the freezer and placed on a bench where they were exposed to ambient light. Inflorescences were incubated on the bench for seven days at room temperature. After the seven day incubation period, *Botrytis* had sporulated on infected berries. The number of *Botrytis* infected and non-infected berries in each inflorescence was recorded using a dissector microscope. Berries were scored as infected if characteristic conidiophores bearing bunch like conidia were present.

7.3 Results and discussion

7.3.1 Downy mildew on inflorescences

Both Brotomax® and Copper caused significant reduction in downy mildew severity on inflorescences relative to the control but were not as effective as the standard fungicide treatments, copper oxychloride and phosphorus acid (Table 7.1).

Table 7.1 Downy mildew severity on inflorescences. The median percent infection per inflorescence was calculated for each plot prior to analysis by ANOVA. *n*=4

Treatment	Flowers infected per inflorescence (%)
Control	20.33 a
Brotomax®	6.18 b
Chitosan	7.14 b
Copper oxychloride	1.40 c
Phosphorous acid	0.19 c

Values within a column followed by the same letter are not significantly different at the 5% level.

7.3.2 Downy mildew control on leaves

Both Brotomax® and chitosan controlled disease on leaves as effectively as copper oxychloride (table 7.2). The phosphorous acid treatment had the lowest disease but was only significantly lower than the Brotomax® and copper oxychloride treatments but not the chitosan treatment.

Table 7.2 Downy mildew severity on shoots. The median percent infection was calculated for each plot prior to analysis by ANOVA. *n*=4

Treatment	Mean downy mildew severity per shoot (%)
Control	32.74 a
Brotomax®	5.53 b
Chitosan	2.39 bc
Copper	5.14 b
Phosphorous acid	0.005 c

Values within a column followed by the same letter are not significantly different at the 5% level.

7.3.3 Botrytis on bunches

Botrytis severity on bunches was not significantly different between the control and any of the elicitor or fungicide treatments after moist incubation in the laboratory (table 7.3). The lack of protection of flower tissue from *Botrytis* in this work by chitosan is in contrast to work with *in vitro* plantlets which were protected against *Botrytis* attack by foliar application of a chitosan solution (Ait Barka *et al.* 2004).

Table 7.3 *Botrytis* severity on bunches following field inoculation. *n*=4.

Treatment	*Botrytis* severity on bunches (%)
Control	51.4 ab
Brotomax®	44.6 b
Chitosan	54.4 ab
Copper oxychloride	47.7 b
Phosphorous acid	62.1 a

Values within a column followed by the same letter are not significantly different at the 5% level.

7.4 Conclusions

Both Brotomax® and chitosan controlled downy mildew under field conditions at flowering, a high risk time for downy mildew infection. Inoculation of leaves and inflorescences ensured there was significant disease pressure during the trial. The disease severity on leaves treated with both Brotomax® and chitosan was not significantly different to the standard protectant fungicide (copper oxychloride). However, the efficacy of Brotomax® and chitosan against downy mildew on inflorescences at flowering was significantly lower than both copper oxychloride and phosphorous acid fungicide treatments. None of the products tested in this trial, including the standard downy mildew fungicides, reduced the severity of *Botrytis* infection on inoculated bunches. Thus, chitosan and Brotomax® are able to effectively control downy mildew in the field but are ineffective against *Botrytis*.

8 Conclusions and Recommendations

Brotomax®, $AlCl_3$ and chitosan have high levels of efficacy against downy mildew, were shown to have contact fungicide activity and were toxic to zoospores *in vitro*. There was some evidence of systemic activity for Brotomax® and $AlCl_3$ for leaf tissue, consistent with an induced resistance response. $AlCl_3$ had high efficacy against downy mildew but caused considerable phytotoxicity and was not studied further. Systemic protection resulted only in disease reductions of approximately 50% while contact fungicide activity could reduce disease 70-100%. However, there was no protection against disease on unsprayed flower tissue when plants were sprayed with inducers on the foliage. No systemic disease protection was observed for chitosan suggesting that it acts solely as a contact fungicide or possibly via local acquired resistance (LAR). No increases in chitinase or β 1,3-glucanase activity, common markers of an induced resistance response, were observed following any elicitor treatment.

Brotomax® and chitosan had similar efficacy in both field conditions and in glasshouse trials. They were as effective as the standard protectant fungicide, copper oxychloride in most trials for controlling foliar disease but protection on developing bunches was significantly lower than the copper fungicide and the systemic fungicide, phosphorous acid.

Neither Brotomax® or chitosan showed any activity against *Botrytis* bunch rot when applied in the field. While inducers were not specifically tested against powdery mildew, powdery mildew at times developed on plants treated with Brotomax® and chitosan in glasshouse trials. Thus, this would suggest that these products do not have strong activity against powdery mildew. Brotomax® and chitosan appeared only to be able to control downy mildew and other fungicide treatments for powdery mildew and *Botrytis* control would still be required. Other inducers reported in the literature to have activity against grapevine downy mildew were not found to be effective in this study.

Brotomax® was as effective as the standard protectant fungicide (copper oxychloride) in some trials. Brotomax® is currently being marketed for viticultural use in Australia as a foliar nutrient formulation. Regular applications of Brotomax® for downy mildew control at the dose rates required would be uneconomic compared to currently available protectant fungicides. However, depending on the rate of Brotomax® used, a foliar application of Brotomax® for nutritional purposes should provide a high level of protection against downy mildew and could potentially replace a downy mildew fungicide application. The activity of Brotomax® may be mainly due to its copper content and a similar result may be able to be achieved much more cheaply by using reduced rates of a copper fungicide.

Chitosan also showed high efficacy against downy mildew and is likely to have no environmental problems due to its non-toxic and biodegradable nature. However, there are currently no chitosan containing fungicides registered for use in Australian. Cost

would be the major obstacle to producing a chitosan product for use in agriculture. Discussions with a local manufacturer of chitosan products have indicated that a formulated agricultural product (5 % (w/v) chitosan) could be produced at a cost of approximately $15/L. This would be less than double the cost of the most expensive fungicides currently available for downy mildew control on a per hectare basis (Appendix 2). Chitosan is currently only commercially produced from crustacean waste and squid. However, chitin, the natural polymer from which chitosan is derived, is the second most abundant biopolymer in nature, second only to cellulose. Current research into producing chitosan from alternative sources such as fungal cell walls may lead to cheaper chitosan in future. Another way the cost of treatment could be reduced is through different spray application technology. Recirculating sprayers, although not widely used in Australia, have been developed overseas where spray drift and run-off are major concerns. This technology would be particularly beneficial early in the season when grapevine canopies are small and the risk of downy mildew infection is highest. If these technologies could be adapted for use in Australian vineyards, the efficiency of spray deposition would be dramatically increased which may allow more expensive but environmentally friendly crop protection products such as chitosan to be used.

Recent Australian research to evaluate the potential of chitosan to enhance plant defence in agriculture has shown promising results for powdery mildew control in tomatoes (Walker *et al.* 2004). This report recommended the further development of chitosan as a crop protection product, especially in *Solanaceae* crops. These results, together with the efficacy against downy mildew described in this report, indicate there is potentially a large market and economies of scale if an agricultural chitosan were to be registered and marketed in Australia. Due to the restrictions on the use of systemic fungicide and the known non-target impacts of some protectant downy mildew fungicides, chitosan has potential to be developed further as a fungicide product for use against downy mildew.

Potassium bicarbonate was only identified as being effective against downy mildew late in the project. Therefore it was not extensively tested and no field trials were conducted. None the less, it did give a high level of disease protection in the glasshouse (99%). It is worth investigating further for the following reasons:

- food grade material
- IPM compatible
- an allowable input in organic farming systems
- already has APVMA registration for grapevines for powdery mildew control (Ecocarb®)
- cost is comparable to other downy mildew fungicides.

Appendix 1. References

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Appendix 2: Comparison of approximate fungicide costs

Product	Cost L or Kg ($)	Rate (L or Kg)/Ha*	Cost/Ha ($)*
Copper oxychloride	3.84	2.5	10
Phosphorous acid	2.42	3.0	7
Mancozeb 750	8.15	3.0	24
Chlorothalanil 720	18.10	3.0	54
Amistar WG®	348.93	0.5	174
Ridomil Gold Plus®	53.50	2.25	120
Chitosan liquid (5%)[1]	15.00	20.0	300
RAGE® chitosan	55.00	40.0	2200
Aminogro®	7.60	5.0	38
Brotomax®	33.00	10.0	330
Ecocarb®	13.35	4.0	53

[1] Product not currently on the market, estimated price supplied by manufacturer

* Assuming 1000 L/Ha application volume, where rate is expressed as a range on the label, the maximum rate was used in the calculation

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