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Inhibition of *Botrytis cinerea* growth and suppression of botrytis bunch rot in grapes using chitosan

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Chitosan inhibited growth of *Botrytis cinerea* in liquid culture and suppressed grey mould on detached grapevine leaves and bunch rot in commercial winegrapes. Germination of *B. cinerea* was completely inhibited in malt extract broth containing chitosan at concentrations greater than 0·125 g L^{-1}. However, treated conidia were able to infect detached Chardonnay leaves and pathogenicity was not affected, even after incubation for 24 h in chitosan at 10 g L^{-1}. When added after conidial germination, chitosan inhibited *B. cinerea* growth and induced morphological changes suggestive of possible curative activity. The effective concentration of chitosan that reduced mycelial growth by 50% (EC_{50}) was 0·06 g L^{-1}. As a foliar treatment, chitosan protected detached Chardonnay leaves against *B. cinerea* and reduced lesion diameter by up to 85% compared with untreated controls. Peroxidase and phenylalanine ammonia-lyase activities were also induced in treated leaves. In vineyard studies, Chardonnay winegrapes exhibited 7·4% botrytis bunch rot severity at harvest in 2007 after treatment with an integrated programme that included chitosan sprays from bunch closure until 2 weeks preharvest, compared with 15·5% in untreated controls and 5·9% with fungicide treatment. In the following season, botrytis bunch rot severity was 44% in untreated Chardonnay at harvest and the integrated programme (21%) was less effective than fungicides (13·8%). However, in Sauvignon blanc winegrapes, the integrated and the fungicide programme each reduced botrytis bunch rot severity to 4% and were significantly different from the untreated control (11·5%). This study provides evidence that suppression of botrytis in winegrapes by chitosan involves direct and indirect modes of action.

Keywords: botrytis bunch rot, *Botrytis cinerea*, chitosan, induced resistance, *Vitis vinifera*, winegrape

Introduction

Botrytis bunch rot or grey mould of grapes is caused by the fungus *Botrytis cinerea* and is one of the most economically important diseases of grapevine (*Vitis vinifera*) worldwide. Floral tissues and immature berries can be infected by *B. cinerea* and the pathogen can remain in a semidormant saprophytic state in necrotic floral tissues or in a latent state within the developing berry (Holz *et al.*, 2003; Keller *et al.*, 2003; Pezet *et al.*, 2003). Localized necrotic scars that arise in the receptacle area following the abscission of calyptra have been identified as important sites for the initial establishment of *B. cinerea*, leading to latent infections (Keller *et al.*, 2003). After veraison, latent infections can resume as the host defences decline and the nutritional content of the ripening berry increases (Pezet *et al.*, 2003). Berry infections also arise directly, particularly in wounded or cracked berries, from conidia and mycelium produced in neighbouring tissues or floral debris trapped within the developing bunch (Nair & Allen, 1993; Holz *et al.*, 2004). Bunch rot can develop rapidly from these infection foci, especially with recurring or prolonged rainfall events, and may spread during the preharvest period, resulting in substantial crop losses at harvest.

Traditionally, management of botrytis bunch rot has relied upon the use of synthetic chemicals applied at specific vine growth stages, including flowering, pre-bunch-closure and post-veraison (Rosslenbroich & Stuebler, 2000). It is not uncommon for fungicides to be applied up to 7 days before harvest, although in New Zealand the preharvest intervals for many fungicides have been extended by limiting the timing of their final application to 80% capfall in order to avoid potential residues in wines. As a consequence, vineyard managers have limited botrytis bunch rot control options after bunch closure and there is increasing demand for botryticides that can be used from veraison until harvest without resulting in residues on the skin at harvest or in the wine.

Chitosan is a naturally occurring polysaccharide derived from chitin that has demonstrated potential to

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control several plant diseases and to extend the storage life of fruits and vegetables (Bautista-Baños *et al.*, 2003, 2006; Meng *et al.*, 2008; Badawy & Rabea, 2009). Several reports have shown that chitosan has antimicrobial activity and can interfere with spore germination and mycelial growth of phytopathogenic fungi, including *Botrytis* spp. (Ben-Shalom *et al.*, 2003; Rabea *et al.*, 2003; Muñoz *et al.*, 2009). Furthermore, chitosan has also been reported to activate plant defences (El-Ghaouth *et al.*, 1994; Aziz *et al.*, 2006; Amborabé *et al.*, 2008) and to enhance plant resistance to fungal infection when applied either as a pre- or as a postharvest treatment (Bautista-Baños *et al.*, 2006; Manjunatha *et al.*, 2008; Nandeeshkumar *et al.*, 2008). Structure–activity studies using purified chitosan oligomers have shown that the degree of polymerization and the degree of acetylation affect their bioactivity (Cabrera *et al.*, 2006; Wang *et al.*, 2006).

Chitosan polymers are non-toxic and biodegradable and are therefore attractive substitutes for synthetic chemical fungicides. A chitosan-based solution, Chitogel® (Ecobulle), was shown to stimulate the growth of grape cv. Chardonnay plantlets and induce resistance to challenge inoculation with *B. cinerea* (Ait Barka *et al.*, 2004). Similarly, treatment of excised grapevine leaves with chitosan caused an elevation of defence-related enzyme activity and induced resistance to infection by *B. cinerea* (Aziz *et al.*, 2006; Trotel-Aziz *et al.*, 2006). In field studies, preharvest applications of 1% chitosan, 21 and/or 5 days before harvest, reduced botrytis bunch rot severity by 55% on table grape cv. Italia compared with untreated controls (Romanazzi *et al.*, 2002). Furthermore, combining preharvest chitosan application with postharvest UV-C irradiation provided greater control of botrytis bunch rot on table grape cvs Autumn Black and B36-55 than either treatment alone (Romanazzi *et al.*, 2006). It was proposed that this integrated strategy could be an alternative to the use of sulphur dioxide for postharvest treatment of table grapes.

The objective of this study was to determine the activity of a water-soluble chitosan concentrate on the germination and growth of *B. cinerea in vitro* and to evaluate its activity against the pathogen on treated Chardonnay grape leaves in the laboratory and against botrytis bunch rot in winegrapes in a commercial vineyard. The effect of chitosan on defence-related enzyme activity in detached Chardonnay leaves was also investigated.

Materials and methods

Compounds

Chitosan was obtained as a liquid concentrate (ARMOUR-Zen® a.i. chitosan 144 g L^{-1}, Botry-Zen Ltd). The liquid concentrate did not contain a wetting agent and so chitosan solutions for detached-leaf assays and field trials were prepared in water containing the non-ionic adjuvant Actiwett (a.i. linear alcohol ethoxylate, Elliot Technologies) to improve wetting, sticking and spreading properties. Chemicals for enzyme studies were obtained from Sigma-Aldrich unless otherwise stated.

Botrytis cinerea inoculum

Botrytis cinerea isolate BCK3 (originally isolated from kiwifruit) was maintained in coolstorage at −70°C in 15% glycerol (v/v) until required. Fresh cultures were grown on oatmeal agar (30 g ground oats, 20 g Bacto agar and 1 L deionised water) in the dark at 18°C for 14 days. Conidia were harvested by flooding the cultures with sterile distilled water containing Tween 20 (0·01% v/v) and then filtering the subsequent suspensions through sterile cell strainers (Falcon, 100-μm mesh) and adjusting the concentration as required with the aid of a haemocytometer.

Effects of chitosan on growth of *B. cinerea*

Growth of *B. cinerea* was measured in 2% malt extract broth (MEB) containing chitosan at 0·0, 0·016, 0·032, 0·0625, 0·125 or 0·25 g L^{-1}. The suspensions contained 1×10^5 *B. cinerea* conidia mL^{-1} and were dispensed as 200-μL aliquots into eight replicate wells of a 96-well microtitre plate. The plate was incubated at 18°C and growth of *B. cinerea* was monitored regularly over a 72-h period by measurement of optical density (OD) at 490 nm using a BioTek PowerWave XS multiwell plate reader (BioTek Instruments Inc.). Assays were also conducted to determine the effect of chitosan on *B. cinerea* growth when added post-germination. A conidial suspension containing 1×10^5 conidia mL^{-1} in 2% MEB was dispensed in 180-μL aliquots into 72 replicate wells in duplicate 96-well microtitre plates. The plates were incubated at 18°C and chitosan was added to selected wells after 24 or 48 h to give final concentrations of 0·0, 0·032, 0·0625, 0·125, 0·25 and 0·5 g L^{-1}. The final volume of each well was 200 μL and there were 12 replicate wells per treatment concentration. Growth of *B. cinerea* was determined by measurement of changes in optical density as described above.

Detached-leaf assays

Effect of chitosan on pathogenicity of conidia

Botrytis cinerea conidia were incubated in chitosan and then used as inocula for infection and pathogenicity studies on detached Chardonnay leaves. Conidial suspensions (1×10^7 conidia mL^{-1}) were incubated for 24 h in 2% MEB containing chitosan at 0·0, 0·625, 1·25, 2·5, 5·0, 10·0 or 20·0 g L^{-1} and then diluted 100× with sterile Milli Q water (Millipore) before inoculation. Young fully expanded grape leaves were removed from potted Chardonnay vines (Mendoza clone on SO4 rootstock) and placed on galvanized metal grids in humidity chambers (35 × 35 × 25 cm) with their petioles immersed in Milli Q water to maintain leaf turgidity. The leaves were lightly misted with Milli Q water and then seven gamma-irradiated necrotic kiwifruit leaf discs (5-mm diameter) were placed at least 30 mm apart onto the adaxial surface of

each grape leaf. The necrotic discs were then inoculated with 7·5 μL of the treated *B. cinerea* conidial suspensions, ensuring that each of the treatment concentrations was represented on each grape leaf. There were 10 replicate leaves. Lesion diameters were measured after 5 days of incubation at 20°C.

Treatment of leaves with chitosan

Fully expanded Chardonnay leaves were removed from potted grapevines and dipped in sterile distilled water containing chitosan at 0, 1·25, 2·5, 5·0, 10·0 or 20·0 g L^{-1} in 0·025% v/v Actiwett®. Excess liquid was removed from leaves by blotting with paper towels and then they were placed on galvanized metal grids in high-humidity chambers as described above, with their petioles immersed in water to maintain leaf turgidity. After 24 h of incubation at 20°C, the leaves were lightly misted with sterile distilled water before placing two gamma-irradiated necrotic kiwifruit leaf discs (5-mm diameter) onto the adaxial surface of each grape leaf. The necrotic discs were positioned symmetrically, with one on either side of the midrib vein and taking care to avoid placement over secondary veins. The necrotic discs were then inoculated with 7·5 μL freshly prepared *B. cinerea* conidial suspension containing 1×10^5 conidia mL^{-1}. There were eight replicate leaves per treatment. Lesion diameters were measured after 5 days of incubation at 20°C. Activities of peroxidase (POX) and phenylalanine ammonia-lyase (PAL) were measured over a 24-h period in Chardonnay leaf discs after treatment with chitosan. Leaf discs (25-mm diameter), from freshly detached Chardonnay leaves, were immersed in sterile distilled water containing 0, 1·25 or 10·0 chitosan g L^{-1} in 0·025% Actiwett®. The leaf discs were lightly blotted with paper towels to dry and then placed on moist Whatman No. 1 filter paper (Whatman Ltd) in plastic trays. The trays were sealed and then incubated at 20°C under continuous light (140 μmol m^{-2} s^{-1}). Three pairs of discs per chitosan concentration were sampled after 0, 4, 8, 12, 18 and 24 h and prepared for enzyme extraction as described previously (Reglinski *et al.*, 2005). PAL activity was measured using L-[U-^{14}C] phenylalanine (Sigma Aldrich) as a substrate using the method of Bernards & Ellis (1989). POX activity was measured using the method described by Polle *et al.* (1990). Protein content of the crude extracts was determined using Bio-Rad protein assay reagent, based on the method of Bradford (1976).

Field trials

Field trials were conducted over two seasons in commercial vineyards with a history of high crop loss from botrytis bunch rot, located in the Hawke's Bay region of New Zealand. In each trial, chitosan was applied during the mid- and late season following early season applications of the biological control agent (BCA) *Ulocladium oudemansii* (BOTRY-Zen®, Botry-Zen Ltd). Early season describes the period from the beginning of flowering until berries are pea-sized, mid-season is the period commencing at pre-bunch-closure, and late season the period commencing at veraison.

In 2006/07, the trial was conducted on 8-year-old Chardonnay vines (clone UCD15 grafted onto 3309 rootstock) that were planted in rows spaced 2·8 m apart with each vine 3·5 m apart within the rows. Four botrytis bunch rot management spray programmes were evaluated: (i) an untreated control, (ii) *U. oudemansii* during the early season, (iii) *U. oudemansii* early season then chitosan mid- and late season, and (iv) synthetic fungicides applied early, mid- and late season. The fungicides were: tolylfluinid (Euparen® Multi, Bayer Crop Science), cyprodinil + fludioxonil (Switch®, Syngenta Crop Protection), captan (Captan FLO, Nufarm) and iprodione (Rovral® FLO, Bayer Crop Science). There were five replicate bays each containing four vines for each treatment laid out in a randomized complete block design. Spray programmes commenced at 5% capfall on 18 November 2006. All vines were sprayed with penconazole (Topas® 200 EW, Syngenta Crop Protection) on 12 December 2006 and with sulphur (Kumulus® DF, BASF Crop Protection) on 20 December 2006 and 12 and 24 January 2007 to control powdery mildew caused by *Erysiphe necator*.

Two vineyards trials were conducted in 2007/08, one in the same Chardonnay vineyard as 2006/07 and another on 18-year-old Sauvignon blanc vines (mass-selected clone grafted onto SO4 rootstock). The Sauvignon blanc rows were spaced 3·0 m apart with each vine 1·4 m apart within the row. At each site, four spray programmes were evaluated: (i) an untreated control, (ii) *U. oudemansii* during the early season with cyprodinil + fludioxinil at pre-bunch-closure and chitosan thereafter, (iii) *U. oudemansii* early season then chitosan, and (iv) synthetic fungicides applied early, mid- and late season. There were six replicate plots for each treatment, laid out in a randomized complete block design with a buffer plot at each end of the row. All vines were sprayed with penconazole on 24 December 2007 and 14 and 23 January 2008 to control powdery mildew.

All treatments were applied using a motorized spray pump and handgun at an application rate of 500 L ha^{-1}. Treatment plots were contiguous single bays, with the first and last metre of each plot used as a buffer zone. The details for each programme are shown in Table 1.

Botrytis bunch rot assessment

Botrytis bunch rot assessment in the 2006/07 trial was carried out on 4 April 2007 and in the 2007/08 trial on 26 March and 18 April 2008. Bunch rot incidence and severity (percentage of bunch area infected) were determined on each of 50 bunches per plot.

Statistical analysis

Analysis of variance was performed using GenStat, 10th edition (Lawes Agricultural Trust). The laboratory data were log-transformed and the field data were

Table 1 Botrytis bunch rot management programmes in 2006/07 and 2007/08

2006/07 Vine phenology	Date	Fungicide	BCA[a]	BCA/chitosan[b]
5% capfall	18 Nov.	Tolylfluinid[c]	*U. oudemansii*	*U. oudemansii*
80% capfall	28 Nov.	Cyprodinil + fludioxonil[d]	*U. oudemansii*	*U. oudemansii*
Berries pea-size	14 Dec.	Captan[e]	*U. oudemansii*	*U. oudemansii*
Pre-bunch-closure	20 Dec.	Cyprodinil + fludioxonil	–	Chitosan
Bunches closed	12 Jan.	Captan	–	Chitosan
Post-bunch-closure	24 Jan.	Captan	–	Chitosan
Veraison	12 Feb.	Captan	–	Chitosan
Post-veraison	22 Feb.	Captan	–	Chitosan
Post-veraison	7 Mar.	Captan	–	Chitosan
Preharvest	22 Mar.	Iprodione[f]	–	Chitosan
2007/08 Vine phenology	**Date[g]**	**Fungicide**	**BCA/fungicide/chitosan**	**BCA/chitosan**
10% capfall	5(4) Dec	Tolylfluinid	*U. oudemansii*	*U. oudemansii*
95% capfall	15(14) Dec.	Cyprodinil + fludioxonil	*U. oudemansii*	*U. oudemansii*
Berries pea-size	28(27) Dec.	Captan	*U. oudemansii*	*U. oudemansii*
Pre-bunch-closure	9 Jan.	Cyprodinil + fludioxonil	Cyprodinil + fludioxonil	Chitosan
Bunches closed	25 Jan.	–	Chitosan	Chitosan
Post-bunch-closure	10 Feb.	Captan	Chitosan	Chitosan
Veraison	23 Feb.	Captan	Chitosan	Chitosan
Post-veraison	10(12) Mar.	–	Chitosan	Chitosan
Preharvest	20(21) Mar.	–	Chitosan	Chitosan
Preharvest[h]	4 Apr.	–	Chitosan	Chitosan

[a]BCA (biological control agent) = BOTRY-Zen® (a.i. not less than $2{\cdot}5 \times 10^8$ CFU g^{-1} *Ulocladium oudemansii*) applied at 4 kg ha^{-1}.
[b]In 2006/07 the chitosan concentrate contained 28 g L^{-1} chitosan and was applied at 25 L ha^{-1}. In 2007/08, the concentrate contained 144 g L^{-1} chitosan and was applied at 5 L ha^{-1}.
[c]Euparen® Multi (a.i. 500 g kg^{-1} tolylfluinid) applied at 2 kg ha^{-1}.
[d]Switch® (a.i. 375 g kg^{-1} cyprodinil, 250 g kg^{-1} fludioxonil) applied at 800 g ha^{-1}.
[e]Captan FLO (a.i. 480 g L^{-1} captan) applied at 2 mL L^{-1}.
[f]Rovral® FLO (a.i. 255 g L^{-1} iprodione) applied at 3 L ha^{-1}.
[g]Treatment dates for Chardonnay, with Sauvignon blanc dates in parenthesis if different.
[h]Chardonnay only.

angular-transformed when necessary to equalize the variance and to better meet the normality assumptions of the analysis. Bias-corrected back-transformed means and standard errors are presented, with the significance from the analyses using transformed data. Logistic regression response curves were fitted to the log-transformed chitosan concentrations in GenStat to obtain the EC_{50} values for *B cinerea* growth rate. Data are presented with standard error of means (SEM) and the standard error of differences of means (SED) is included where appropriate.

Results

Effect of chitosan on growth of *B. cinerea* conidia *in vitro*

Growth of *B. cinerea* was significantly inhibited ($P < 0{\cdot}001$) when incubated in liquid media containing chitosan at concentrations greater than or equal to 0·016 g L^{-1} (Fig. 1a). The effective concentration of chitosan that reduced growth by 50% (EC_{50}) was 0·03 g L^{-1}, as calculated by non-linear regression analysis of the data recorded at 72 h. No measurable growth was recorded over the 72-h period when conidia were incubated in chitosan at 0·125 or 0·25 g L^{-1}. Conidia resumed growth after being washed and transferred to fresh MEB (data not shown). Chitosan also inhibited the growth rate of *B. cinerea* when added to actively growing cultures in MEB after 24 or 48 h (Fig. 1b,c). Cultures were more sensitive to chitosan if treated after 24 h of incubation than after 48 h. For example, treatment with 0·125 g L^{-1} chitosan significantly ($P < 0{\cdot}05$) retarded growth when added to cultures after 24 h of incubation, but not after 48 h. The EC_{50} values, as calculated by non-linear regression analysis of the growth rate after chitosan addition, were 0·06 and 0·175 g L^{-1} for cultures treated after 24 and 48 h, respectively. Growth inhibition of *B. cinerea* was accompanied by cytological changes, including cytoplasmic granulations, retraction of cytoplasm and shrinkage of the mycelium (Fig. 2).

Detached-leaf assays

Effect of chitosan on the pathogenicity of B. cinerea *conidia*

Botrytis cinerea conidial suspensions retained an ability to infect detached Chardonnay leaves even after incubation for 24 h in MEB supplemented with chitosan at concentrations up to 10 g L^{-1} (Table 2). However,

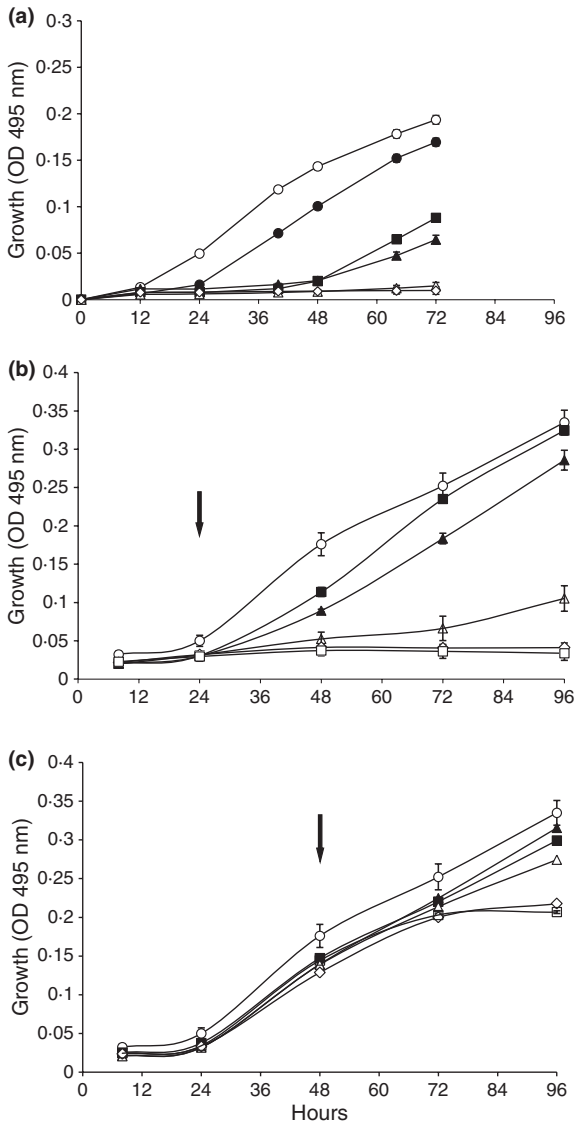

Figure 1 Growth of *Botrytis cinerea* in 2% malt extract broth (MEB) amended with chitosan after 0 (a), 24 (b) or 48 h (c). Black arrows indicate the 24- and 48-h additions of chitosan. Final chitosan concentration was 0 (○), 0·016 (•), 0·032 (■), 0·0625 (▲), 0·125 (Δ), 0·25 (◇) or 0·5 g L^{-1} (□). Data are presented as means ± SEM.

pathogenicity of treated conidia was significantly inhibited (P = 0·002) after incubation in chitosan at 20 g L^{-1}, as evidenced by a 72% reduction in grey mould leaf lesion diameter 120 h after inoculation. Sporulation was observed on leaf lesions in all treatments but was not recorded.

Treatment of detached leaves with chitosan

Detached Chardonnay leaves that were treated with chitosan 24 h before inoculation with *B. cinerea* exhibited greater resistance to infection than leaves that were treated with Actiwett® alone (Fig. 3). Five days after inoculation, grey mould lesion diameter on leaves treated with chitosan at concentrations equal to or greater than 5 g L^{-1} were significantly smaller (P < 0·001) than lesions on the Actiwett® control leaves. Activities of POX and PAL were induced by chitosan in Chardonnay leaf discs (data not shown). POX activity was elevated (P = 0·045) after 18 h in leaves treated with chitosan at 1·25 or 10 g L^{-1} when compared with Actiwett® controls. PAL activity was elevated (P = 0·05) from between 4 and 18 h after treatment with 10 g L^{-1} chitosan compared with the Actiwett® controls.

Field trials

Botrytis bunch rot incidence at harvest in 2006/07 was 47·6% in untreated Chardonnay winegrapes and bunch rot severity was 15·5% (Table 3). Three applications of *U. oudemansii* during the early season reduced bunch rot incidence to 30% and severity to 11·5%. Disease control was enhanced further when early season applications of *U. oudemansii* were followed by applications of chitosan between bunch closure and harvest. This integrated programme reduced botrytis bunch rot incidence to 11·3% and severity to 7·4% and was not significantly different to the full fungicide programme.

In 2007/08, botrytis bunch rot incidence in the untreated controls at harvest was 84·2% in Chardonnay and 67·3% in Sauvignon blanc (Table 4). Average botrytis bunch rot severity in these two cultivars was 44·1 and 11·5%, respectively. Each of the botrytis spray programmes significantly reduced bunch rot when compared with the untreated control in both cultivars. In Sauvignon blanc, the BCA/chitosan and BCA/fungicide/chitosan programmes each reduced severity to 4% or less and were as effective as the full fungicide programme (4·3% severity). In Chardonnay, the BCA/fungicide/chitosan programme reduced severity to 11·8% which was equivalent to the full fungicide programme (13·8%) and significantly more effective than BCA/chitosan (21·4%).

Discussion

There is increasing interest in the use of BCAs and natural products, such as chitosan, in plant disease management. Chitosan is a naturally occurring polysaccharide with demonstrated potential as a disease control agent on account of its inhibitory activity against several pathogenic fungi (Rabea *et al.*, 2003; Xu *et al.*, 2007) and its capacity to stimulate plant defence mechanisms (Aziz *et al.*, 2006; Trotel-Aziz *et al.*, 2006). In this study, water-soluble chitosan was evaluated for activity against *B. cinerea* and its potential to control botrytis bunch rot in winegrapes. Chitosan directly inhibited growth of *B. cinerea* in liquid culture and reduced grey mould lesion development when applied to detached Chardonnay leaves before pathogen inoculation. In vineyard trials, repeated spray applications of chitosan after pre-bunch-closure, integrated with early season applications of a BCA (*U. oudemansii*), significantly reduced the incidence

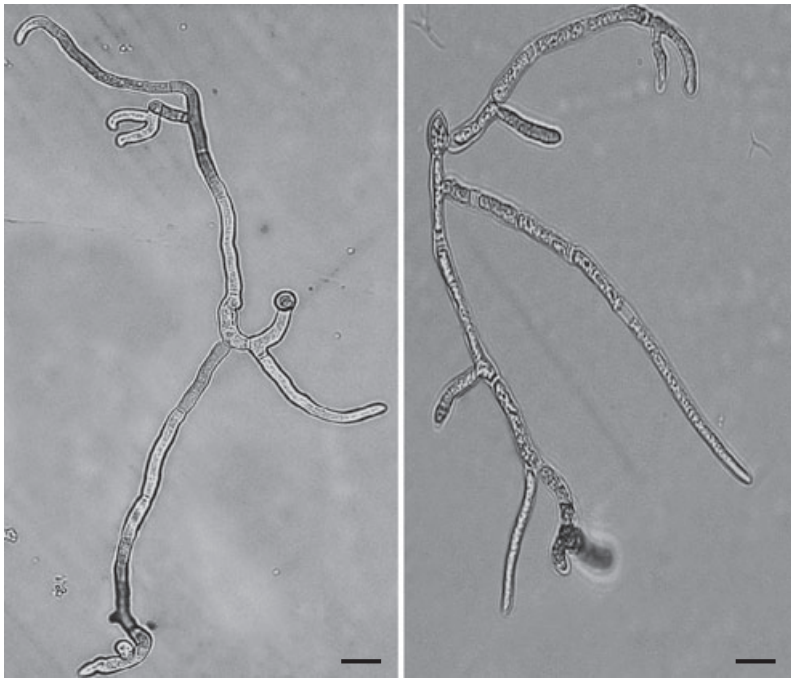

Figure 2 *Botrytis cinerea* mycelium in 2% malt extract broth (MEB) after 24 h (left), and then 7 h later (right), following addition of chitosan at 1·4 g L^{-1}. (Bar = 10 μm).

and severity of botrytis bunch rot in Chardonnay and Sauvignon blanc winegrapes.

Chitosan-based formulations have demonstrated activity against *B. cinerea* (Ait Barka *et al.*, 2004; Aziz *et al.*, 2006; Trotel-Aziz *et al.*, 2006) and downy mildew (Aziz *et al.*, 2006) on grape plantlets and have proven effective against botrytis bunch rot on table grapes when applied as a preharvest spray (Romanazzi *et al.*, 2002, 2006) or as a postharvest dip (Romanazzi *et al.*, 2002, 2007). In this study, there was a dose-dependent inhibition of *B. cinerea* germination and growth in media containing chitosan at concentrations above 0·016 g L^{-1}, with complete growth suppression occurring at 0·125 g L^{-1} (EC_{50} 0·03 g L^{-1}). These concentrations are comparable with those reported as being inhibitory to *B. cinerea* in previous studies (Ben-Shalom *et al.*, 2003; Trotel-Aziz *et al.*, 2006). Treated conidia resumed growth when washed and transferred into fresh media, indicating that chitosan is fungistatic rather than fungicidal. Chitosan also exhibited inhibitory activity when added to conidial suspensions after germination although the concentration of chitosan required to arrest mycelial growth was greater than that required to prevent conidial germination. Microscopy revealed that *B. cinerea* developed granular inclusions in the cytoplasm and that mycelium appeared to contract following the addition of chitosan. Similar morphological changes were observed in *B. cinerea* after treatment with chitogel (a.i. chitosan, Ecobulle) (Ait Barka *et al.*, 2004) and this is consistent with the suggestion that chitosan interferes with cell permeability, leading to the subsequent leakage of intracellular electrolytes and proteinaceous material (Xu *et al.*, 2007). Mycelial growth was less sensitive to chitosan when suspensions were treated after 48 h incubation (EC_{50} 0·175 g L^{-1}) than after 24 h (EC_{50} 0·06 g L^{-1}), suggesting that chitosan has curative activity against *B. cinerea* but that this declines as the pathogen develops. The basis for this decline in sensitivity is not known, but may, in part, be the result of 'detoxification' of chitosan via the action of cellulases and pectinases commonly associated with *B. cinerea* pathogenesis (Kars & van Kan, 2004). Purified forms of these enzymes have been shown to exhibit non-specific hydrolytic activity against chitosan leading to its degradation (Xia *et al.*, 2008).

Table 2 Mean lesion diameter on detached grape cv. Chardonnay leaves measured 120 h after inoculation with chitosan-treated *Botrytis cinerea* conidial suspensions

Chitosan (g L^{-1})	Lesion diameter (mm)
0	13·1
0·625	15·0
1·25	13·7
2·5	14·2
5	16·6
10	8·1
20	3·9

SED = 3·72, P = 0·002. Data are back-transformed means.

Detached-leaf studies indicated that pathogenicity of *B. cinerea* on Chardonnay leaves was not affected when conidia were incubated for 24 h in chitosan at 10 g L^{-1}

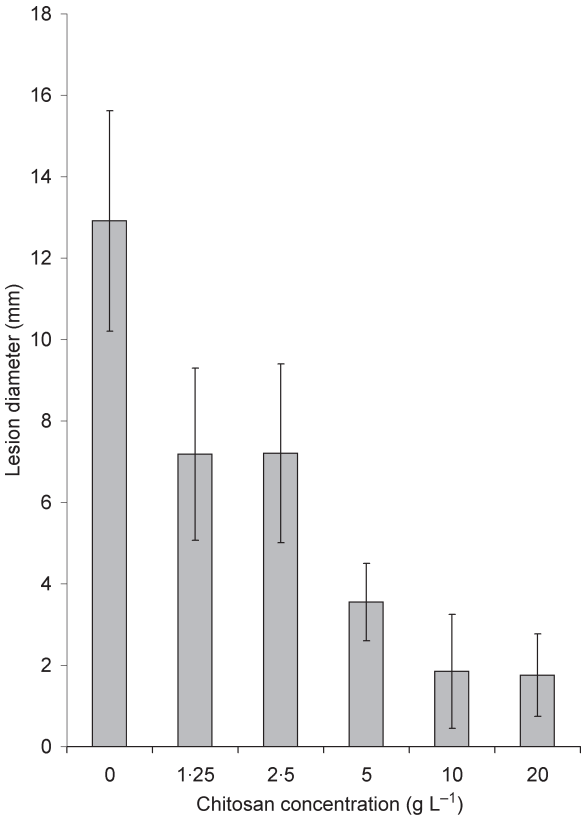

Figure 3 Mean lesion diameter on detached grape leaves treated with chitosan 24 h before challenge inoculation with *Botrytis cinerea*. Lesion diameters were measured 120 h after inoculation. Data are presented as means ± SEM. SED = 2·62, $P < 0·001$.

Table 3 Botrytis bunch rot incidence and severity at harvest on 4 April 2007 in grape cv. Chardonnay

Spray programme	Incidence (%)	Severity (%)
Untreated	47·6	15·5
Fungicide	6·6	5·9
BCA[a]	30·0	11·5
BCA/chitosan	11·3	7·4
SED	7·8	3·1
FProb	<0·001	<0·05

Data are back-transformed means.
[a]BCA (biological control agent) = *Ulocladium oudemansii*.

before inoculation. This concentration is approximately 40 times greater than that shown to prevent conidial germination and supports the notion that chitosan is fungistatic. It is postulated that germination and leaf infection occurred once the 'local' concentration of chitosan fell below an inhibitory level. Interestingly, chitosan was more effective against grey mould leaf infections when applied to the leaf before inoculation than when applied directly to the inoculum. Moreover, chitosan stimulated POX and PAL in detached leaves, suggesting that induced resistance may contribute to the suppression of grey mould in grape leaves. Peroxidases are involved in cell wall fortification, whilst PAL is a key enzyme in the biosynthesis of defence-related phytoalexins and phenolics in grapes. These results are consistent with earlier demonstrations of enzyme induction and elevation of resistance to *B. cinerea* obtained using chitosan on Chardonnay plantlets (Trotel-Aziz *et al.*, 2006). Mechanisms responsible for defence activation by chitosan remain to be fully determined, but there is evidence that the response may be initiated by a chitosan-induced depolarization of the plasma membrane (Amborabé *et al.*, 2008). Early perception of fungi by plants is triggered via an elicitor-induced membrane depolarization and this event leads to the activation of inducible host defences (Zipfel, 2008).

In vineyard studies, applications of the *B. cinerea* antagonist *U. oudemansii* in the early season followed by regular treatment with chitosan from bunch-closure until harvest significantly reduced botrytis bunch rot on Chardonnay and Sauvignon blanc winegrapes. This integrated BCA/chitosan spray programme reduced botrytis bunch rot severity from 15·5% (in the untreated plots) to 7·4% on Chardonnay in 2006/07 and from 11·5% (untreated) to 4% on Sauvignon blanc in 2007/08 and was as effective as the synthetic fungicide programme. However, the BCA/chitosan programme was less effective than the synthetic fungicide programme on Chardonnay in 2007/08, when there was greater disease pressure in this vineyard as evidenced by 44% botrytis bunch rot severity in untreated controls, compared with 15·5% severity in the previous season. The mode of action of chitosan in grape berries was not determined, but, based on laboratory studies, it is postulated that disease control resulted from a combination of direct antifungal activity and elevation of host resistance. Previous studies on table grapes (Romanazzi *et al.*, 2006; Meng *et al.*, 2008) and in winegrapes (Duxbury *et al.*, 2004) found that preharvest spray application of chitosan did not affect berry phenolics, suggesting that these chemical defences are not critical in explaining the activity of chitosan against bunch rot. However, Meng *et al.* (2008) also reported that preharvest application of chitosan stimulated peroxidase activity in table grapes, and this may indicate that physical defences play an important role in disease resistance, since this enzyme can catalyse cross-linking reactions that fortify plant cell walls. It is possible that chitosan itself provides a physical barrier to infection since it has been shown that chitosan can form a film on the surface of berries (Romanazzi *et al.*, 2002, 2006). However, the effectiveness of chitosan films against postharvest grey mould on table grapes did not correlate with the viscosity or thickness of the chitosan film and the authors proposed that disease control was attributable to antifungal and elicitor activity (Romanazzi *et al.*, 2009). Since grape berries become more susceptible to infection as they mature, because of a decline in natural defences (Elmer & Reglinski, 2006), it is likely that the antifungal activity of chitosan will become increasingly important for the control of botrytis bunch rot as the berries ripen.

Table 4 Botrytis bunch rot incidence and severity at harvest in Sauvignon blanc and in Chardonnay winegrapes in March and April 2008

	Sauvignon blanc (26 March 2008)		Chardonnay (18 April 2008)	
Spray programme	Incidence (%)	Severity (%)	Incidence (%)	Severity (%)
Untreated	67·3	11·5	84·2	44·1
Fungicide	12·5	4·3	30·5	13·8
BCA[a]/fungicide/chitosan	6·2	1·6	24·8	11·8
BCA/chitosan	23·3	4·0	52·3	21·4
SED	6·9	2·2	7·0	1·9
FProb	<0·001	0·005	<0·001	<0·001

Data are back-transformed means.
[a]BCA (biological control agent) = *Ulocladium oudemansii.*

This study provides evidence that chitosan offers a dual mode of action against botrytis bunch rot in winegrapes, involving direct antifungal activity and induction of natural plant defences. Chitosan was effective when used as a component of an integrated management programme, but less effective than the fungicide programme under high disease pressure. Because chitosan has low mammalian toxicity and is biodegradable it represents a viable alternative to synthetic fungicides, which can result in chemical residues on grapes at harvest and subsequently in wines (Gabriolotto *et al.*, 2009). It would be useful to establish whether more frequent use or higher application rates of chitosan could provide greater control under high disease pressure conditions in susceptible cultivars. Alternatively, it may be appropriate to consider timing of chitosan application relative to identified infection periods (Broome *et al.*, 1995), rather than according to grape phenology. This may enable the treatment to delay conidial germination when environmental conditions are favourable for infection. Future research should also consider potential effects of chitosan on pathogen fitness since, although sporulation was observed in chitosan-treated tissues, conidial production and pathogenicity was not quantified. It is likely that the observed reduction in infections (leaf lesions or bunch infections) would cause a decrease in inoculum production and thereby suppress disease development. Nevertheless, new management measures will be acceptable only if their efficacy is durable over time. Therefore, it would be prudent to evaluate the potential threat of selection for resistance to chitosan amongst *B. cinerea* populations.

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