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Potential use of chitosan in the control of grapevine trunk diseases

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Summary. Due to its fungistatic or fungicidal potential, chitosan, a high molecular-weight polymer that is non-toxic and biodegradable, has become an alternative to conventional fungicides. In addition, chitosan is reported to elicit defense mechanisms in plant tissues. In this study, we explored the *in vitro* fungicidal effect of chitosan on some of the most important grapevine wood fungi, such as *Botryosphaeria* sp. (dieback and cane blight), *Phomopsis* sp. (Phomopsis cane and leaf spot), *Eutypa lata* (eutypa dieback), *Neonectria liriodendri* (black foot disease), *Phaeomoniella chlamydospora* (Petri disease and esca) and *Fomitiporia* sp. (esca). Inhibition of mycelial growth was evaluated at five concentrations 50, 25, 5, 2.5 and 0.5 mg a.i. l^{-1} of chitosan. Chitosan was effective in reducing mycelial growth of all the fungi. The lowest EC_{50} values were obtained with *Pa. chlamydospora, Fomitiporia* sp. and *Botryosphaeria* sp., and the highest with *Neon. liriodendri*. All these were inferior to the maximum recommended field rate (8.33 mg a.i. l^{-1}) with exception of the value obtained with *Neon. liriodendri*. Greenhouse experiments were carried out to evaluate the efficacy of foliar sprays of chitosan on potted grapevine plants (cultivar Castelão) growing in a substrate artificially infested with *Pa. chlamydospora* or *Neon. liriodendri*. The effect of chitosan against *Neon. liriodendri* was similar to that achieved with some selected fungicides (carbendazim+flusilazole, cyprodinil+fludioxonil and tebuconazole). Chitosan significantly improved plant growth (plant height and number of roots) and decreased disease incidence compared with untreated plants. As regards *Pa. chlamydospora,* chitosan only reduced the disease incidence caused by this fungus.

Keywords: fungicides, black foot disease, Petri disease, grapevine-wood fungi.

Introduction

Grapevine trunk diseases are still a threat for viticulturists and nursery operators worldwide and effective control strategies are not yet available for many of them. In Portugal, surveys carried out during the last 10 years in vineyards, nurseries and mother-blocks showed the high incidence and severity of fungi associated with wood diseases and grapevine declines, e.g., *Neonectria liriodendri* (anamorph *Cylindrocarpon liriodendri* [Halleen *et al*., 2006], *Phaeomoniella chlamydospora, Botryosphaeria* spp., *Eutypa* sp. and *Fomitiporia* sp. (Chicau *et al.,* 2000; Rego *et al*., 2000, 2001; Phillips, 2002; Oliveira *et al*., 2004; Sofia *et al*., 2006, Rego *et al*., 2006b). Since many of these fungi are frequently isolated from the same vine, strategies including either conventional fungicides or biopesticides should be directed for different target pathogens, thus providing a wide disease(s) control (Oliveira *et al*., 2004; Fourie and Halleen, 2006).

Chitosan (poly-b-(1,4)-D-glucosamine) is a non-toxic, biocompatible and biodegradable polymer, commercially produced by deacetylation of chitin, one of the most abundant natural compounds found in nature. Chitin is a structural polysaccharide present in the exoskeleton of arthropods, nematodes and in the cell walls of fungi and some algae

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(Rhazi *et al*., 2000; Dahiya *et al*., 2006). Currently, chitin and its derivative chitosan are industrially extracted from shrimps and crab shells as by-products of the seafood industry (Wu *et al*., 2004), but other chitinous sources, such as fungal mycelia, are gaining importance (Teng *et al*., 2001; Wu *et al*., 2005). Due to their unique characteristics, the chitin/chitosan products have wide-range applications in the fields of agriculture, environment, biomedicine, pharmaceuticals, biotechnology and industrial food processing (Hirano, 1999; Synowiecki and Al-Khateeb, 2003). In agriculture, the uses of these biopolymers as food antimicrobials and biopesticides are especially promising (Wu *et al*., 2005). As biopesticide, chitosan has demonstrated anti-fungal and anti-bacterial activities, and has also been found to activate several plant defence responses. The exact mechanisms by which chitosan operates as fungicide are not fully elucidated, but the growth of several plant pathogenic fungi, e.g., *Fusarium* spp., *Puccinia arachidis*, *Colletotrichum gloeosporioides, Botrytis cinerea* is inhibited (Bell *et al*., 1998; Sathiyabama and Balasubramanian, 1998; Bautista-Baños *et al*., 2003; Aït Barka *et al*., 2004).

Chitosan effectively reduced polygalacturonases produced by *B. cinerea* and also induced severe structural alterations in the fungal cells (Aït Barka *et al*., 2004). On the other hand, plants elicited by chitosan prior inoculation showed increased activities of chitosanases and peroxidases and the growth of *B. cinerea* was inhibited. (Ben-Shalom *et al*., 2003). However, the induction of the defence response without the antifungal activity of chitosan was not considered enough to suppress the disease. Also chitinase and glucanase production was stimulated in plants treated with chitosan (Benhamou, 1996), and the synthesis of phytoalexins was induced (Agrawal *et al*., 2002). Tomato plants treated with chitosan showed improved resistance to Fusarium crown and root rot by enhancement of localised cell defenses, as wall appositions, and occlusion of xylem vessels through pits (Benhamou and Theriault, 1992). So, besides fungicidal effect, it is utmost probable that chitosan protects the plants by triggering early plant defence mechanisms. In addition, chitosan is reported as a growth stimulator of plant tissues (Aït Barka *et al*., 2004; Nge *et al*., 2006).

Concerning grapevine wood diseases, chitosan was previously tested against *Pa. chlamydospora, Phaeoacremonium aleophilum* and *Fomitiporia punctata*, but results were not very promising (Bruno *et al*., 2001). However, because of variations in extraction procedures, as well as in the different sources of chitinous material, the functional properties and bioactivity of chitin and chitosan reported from different authors are not reliably comparable (Wu *et al*., 2004).

The objectives of this research were to determine the biological efficacy of chitosan on the mycelial growth of the main fungi associated with grapevine wood diseases and declines, to evaluate its effect on grapevine plants infected with *Neon. liriodendri* and *Pa. chlamydospora* and to compare the results with those obtained for selected fungicides.

Materials and methods

Fungal strains and cultural conditions

The following fungi were used in the *in vitro* studies: *Neon. liriodendri*, *Pa. chlamydospora*, *Botryosphaeria* sp., *Phomopsis* sp. and *Fomitiporia* sp. from the culture collection at the Instituto Superior de Agronomia, Lisbon, Portugal. All were obtained from symptomatic grapevine materials grown in Portugal. An isolate of *Eutypa lata* from INRA, Bordeaux, France was also included (Table 1). Stock cultures of each fungus were maintained on slants of potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) at 5ºC and regularly subcultured on Petri dishes containing PDA, at 24ºC, in darkness.

Effects of chitosan on fungal growth

The chitosan-based biopolymer tested in this assay was Chitosan Oligosaccharin (Gofar Agro, Beijing, China), a liquid formulation of a biological fungicide extracted from marine organisms, with approximately 0.5% a.i. (molecular weight ≤3 kDa) and pH 4–5. Five decreasing concentrations of chitosan (50, 25, 5, 2.5 and 0.5 mg a.i. l^{-1}) were prepared, by adding appropriate volumes to molten PDA medium at 50°C. Aliquots of 15 ml of the chitosan-amended PDA were poured into 9-cm-diameter Petri dishes. After cooling, one mycelial plug (3 mm diameter) cut from the growing edge of each culture, was transferred to the centre of each plate. In control plates sterile distilled water (SDW) was used. For each concentration and fungus, six

Table 1. Grapevine wood fungi isolates used in this study.

Isolate identity	Isolate code	Location	Year of isolation	Grapevine cultivar or rootstock
Phaeomoniella chlamydospora	Pa9	Pinheiro da Cruz, Portugal	2000	Castelão
Neonectria liriodendri	Cy68[a]	Bombarral, Portugal	1999	99R
Eutypa lata	BX 1–10[b]	Bordeaux, France	1990	Cabernet Sauvignon
Fomitiporia sp.	Fmt[c]	Nelas, Portugal	2000	Touriga Nacional
Botryosphaeria sp.	Bt1	Monção, Portugal	2005	Alvarinho
Phomopsis sp.	Phom-04	Alter do Chão, Portugal	2004	Alfrocheiro

[a] CBS117526.
[b] Provided by P. Lecomte, INRA, Bordeaux, France.
[c] Provided by J. Sofia, DRABL, CEVDão, Nelas, Portugal.

replicates were included and all experiments were repeated at least once within 15 days. The dishes were incubated for an adequate period, in the dark, at 24ºC, and the colony diameters were measured. For each concentration of chitosan and fungus, percentages of mycelial growth inhibitions were converted to probits and plotted against $\log_{10}$ values of the chitosan concentration. The probit regression analysis was used to calculate the effective concentration values that inhibited mycelial growth by 50% (EC_{50} values) and their 95% confidence limits values, using STATISTICA 6.0 (StatSoft Inc., Tulsa, OK, USA).

***In vivo* experiments**

The effects of chitosan (Chitosan Oligosaccharin) were evaluated and compared with those achieved by three fungicides in grapevine potted plants (cv. Castelão) grown in soil mixture artificially infested with *Neon. liriodendri* or *Pa. chlamydospora*. Details of the products are provided in Table 2. The fungicides were selected according to their efficacy against *Pa. chlamydospora* (Jaspers, 2001) and *Neon. liriodendri* (Rego *et al*., 2006a).

Neonectria liriodendri was grown in Erlenmeyer flasks containing 250 ml of Czapeck-Dox liquid medium (Modified) (Oxoid Ltd, Basingstoke, UK) for 12 days and *Pa. chlamydospora* in malt extract (2%) (Difco Laboratories) for 14 days. Both cultures were maintained under reciprocal shaking (90 strokes min^{-1}) at room temperature in the dark. After incubation, the cultures were filtered through cheesecloth and the spore suspensions were adjusted to a final concentration of approximately 10^8 spores ml^{-1}. Each spore suspension was added (1:10, v:v) to a mixture of soil, peat and sand (2:1:1, v:v) placed in 1-liter plastic pots. Sterile distilled water (SDW) was used in uninfested controls.

Table 2. Characteristics of fungicides and chitosan tested against *Neonectria liriodendri* and *Phaeomoniella chlamydospora* in *in vivo* experiments.

Active ingredient	Trade name	Company	Concentration a.i.	Rate tested a.i.
Tebuconazole	Folicur WG25	Bayer	25%	100 mg l^{-1}
Cyprodinil+ fludioxonil	Switch 62.5 WG	Syngenta	37.5% cyprodinil+ 25% fludioxonil	375 mg l^{-1}cyprodinil+ 250 mg l^{-1}fludioxonil
Carbendazim+ flusilazole	Escudo	DuPont	10 g l^{-1} carbendazim+ 5 g l^{-1} flusilazole	25 mg l^{-1}carbendazim+ 12.5 mg l^{-1}flusilazole
Chitosan	Chitosan oligosaccharin	Grofar Agro	0.5%	7.14 mg l^{-1}[a]

[a] The field rate recommended by the manufacturer is 6.25 to 8.33 mg a.i. l^{-1}.

Rooted grapevine cuttings cv. Castelão were uprooted and their roots were slightly pruned. The fungicide treatments were carried out by dipping the roots and the basal end of each cutting for 50 min in the fungicide suspensions, before transplantation. The chitosan was applied by foliar spray, after transplantation, and the treatment was repeated one week later. Control plants were similarly treated, but SDW was used instead of fungicide or chitosan. All the products were prepared according to the recommended field rates for other grapevine diseases (Table 2). For each fungus and product, ten replicates were included. Treated and untreated potted grapevine plants were placed at random in a greenhouse, fitted with a cooling system, at 24±5ºC day/18ºC night with natural daylight, and watered and fertilised as required.

After three months, all plants were uprooted and evaluated for the total number of roots and plant height.

Fungal isolations were made from necrotic tissues located within 5 cm of the basal end of the vines and the identity of isolates was confirmed on the basis of morphological characters (Rego *et al*., 2000). The incidence of *Cylindrocarpon*-black foot disease and Petri disease was determined as the mean percentage of grapevine plants that were infected by *Neon. liriodendri* or *Pa. chlamydospora*. Data were subjected to analysis of variance (ANOVA) and treatment means compared by using Tukey's test at a 5% significance level (STATISTICA 6.0). Percentages were transformed to arcsine-square root values before ANOVA.

Results and discussion

Effects of chitosan on fungal growth

In probit regression analyses, coefficients of determination (r^2) were all higher than 0.80 and confidence limits (CL) of EC_{50} were all lower than 0.30 (Table 3). All fungi, but *Neon. liriodendri*, were inhibited by concentrations of chitosan under the maximum field rate (8.33 mg l^{-1}). Three patterns of response were registered being *Pa. chlamydospora* (EC_{50} 1.17 mg l^{-1}), *Fomitiporia* sp. (EC_{50} 1.53 mg l^{-1}) and *Botryosphaeria* sp. (EC_{50} 1.56 mg l^{-1}) isolates the most sensitive to chitosan (Table 3). The sensitivity of *Pa. chlamydospora* and *Fomitiporia* to chitosan was first reported by Bruno *et al*. (2001), who achieved the complete inhibition of the radial growth of both fungi for a concentration of 50 mg l^{-1}. In the present study, however, a similar result was obtained when chitosan was used at one-half of that concentration (data not shown).

Also remarkable is the activity of chitosan against *Botryosphaeria* sp. isolate and further studies should be carried out to test the sensitivity of other *Botryosphaeria* spp. isolates affecting grapevine. An intermediate pattern of sensitivity was detected for *E. lata* (EC_{50} 3.26 mg l^{-1}) and *Phomopsis* sp. (EC_{50} 5.28 mg l^{-1}). Finally, *Neon. liriodendri* appears as the least sensitive fungus to chitosan, with an EC_{50} value (24.65 mg l^{-1}) higher than the concentration recommended for field use on grapevine. This result was not unexpected because data from other authors indicated that the mycelial growth of *C. destructans* from coniferous trees, a close related fungus, was only inhibited 43 and

Table 3. Activity of chitosan against mycelial growth of different grapevine wood fungi assessed by regression analysis.

Fungus	Mycelial growth inhibition		Regression parameters		
	EC_{50} (mg a.i. l^{-1})[a]	CL_{95} (+/-)[b]	Intercept	Slope	r^2 value[c]
Phaeomoniella chlamydospora	1.17	0.14	2.19	2.63	0.96
Fomitiporia sp.	1.55	0.20	1.95	2.56	0.83
Botryosphaeria sp.	1.56	0.30	1.53	2.10	0.85
Eutypa lata	3.26	0.22	1.14	2.55	0.94
Phomopsis sp.	5.28	0.20	1.39	2.10	0.80
Neonectria liriodendri	24.65	0.23	2.39	1.10	0.96

[a] EC_{50}, effective concentration (concentration of chitosan which reduced mycelial growth by 50%).
[b] CL_{95}, confidence limits (95% probability).
[c] r^2, coefficient of determination.

71%, when 1 and 2 g l^{-1} of chitosan were used, respectively (Laflamme *et al*., 1999). Nevertheless, it should be stressed that results from different authors are not reliably comparable, because the biological activity of chitosan depends on the deacetylation degree of chitin (chitosan/chitin balance), the molecular weight and the pH of the culture medium (Alfredsen *et al*., 2004; Wu *et al*., 2004; Torr *et al*., 2005). Further, some discrepancy between our results and those obtained by others might be due to some variability among fungal isolates.

***In vivo* experiments**

The effect of chitosan was evaluated on grapevine potted plants, cv. Castelão, grown in substrate infested with *Neon. liriodendri* or *Pa. chlamydospora*. Concerning *Neon. liriodendri*, chitosan significantly promoted the plant growth and the total number of roots of infected plants, and simultaneously reduced the disease incidence, in comparison with control plants (Table 4). The efficacy of chitosan was equivalent to that achieved by conventional fungicides, tebuconazole and the mixtures cyprodinil+fludioxonil and carbendazim+flusilazole. Previous results already indicated that these fungicides were among the most effective against *Neon. liriodendri*, significantly improving plant growth and health of grapevine plants (Rego *et al*., 2006a). Moreover, a remarkable level of black foot disease control was achieved when these fungicides were used in commercial grapevine nurseries (data not shown).

Although not entirely expected from the *in vitro* assays, chitosan showed efficacy against *Neon. liriodendri* in *in vivo* experiments. This apparent discrepancy between *in vitro* and *in vivo* data is probably a consequence of the further effect of chitosan on triggering a defensive response in treated plants.

Against *Pa. chlamydospora*, the chitosan treatment significantly (α=0.05) reduced fungal colonization (percentage of isolation) compared with the unsprayed controls. No significant differences were observed between chitosan and fungicides. Other parameters under evaluation were not significantly affected by treatments (Table 5). The low infection incidence recorded in control plants (18.3% *Pa.*

Table 4. Effects of chitosan and fungicides on grapevine plants of cv. Castelão (plant height and total number of roots) grown in substrate infested by *Neonectria liriodendri* and on fungal percent isolation.

Treatment	Plant height (mm)[a]	Total number of roots	*Neon. liriodendri* isolation (%)
Water control	105.90 a	34.32 a	80.83 a
Tebuconazole	106.40 ab	44.60 ab	43.33 ab
Carbendazim+flusilazole	112.50 ab	51.30 bc	40.83 ab
Cyprodinil+fludioxonil	141.00 ab	60.20 c	21.67 b
Chitosan	136.90 b	46.35 b	32.05 b

[a] In each column, means followed by the same letter are not significantly different (α=0.05) according to Tukey's test.

Table 5. Effects of chitosan and fungicides on grapevine plants cv. Castelão (plant height and total number of roots) grown in substrate infested by *Phaeomoniella chlamydospora* and on fungal percent isolation.

Treatment	Plant height (mm)[a]	Total number of roots	*Pa. chlamydospora* isolation (%)
Water control	153.20 ab	31.00 a	18.30 a
Tebuconazole	120.80 a	37.70 a	6.70 ab
Carbendazim+flusilazole	176.00 b	43.50 a	11.70 ab
Cyprodinil+fludioxonil	184.90 b	45.50 a	3.70 b
Chitosan	155.50 ab	47.30 a	3.30 b

[a] In each column, means followed by the same letter are not significantly different (α=0.05) according to Tukey's test.

chlamydospora isolation) shows that *Pa. chlamydospora* is not so successful as *Neon. liriodendri* (80.8% isolation) in colonizing grapevine plants from inoculum existing in the soil. Concerning *Pa. chlamydospora*, this finding was previously stated by others (Adalat *et al*., 2000) and in future experiments another method of inoculation (e.g. wound surface inoculation, inoculum injection) should be assayed.

Chitosan showed a promising inhibiting effect on mycelial growth of the main fungi involved with grapevine wood diseases and declines, with emphasis on *Pa. chlamydospora*, *Fomitiporia* sp. and *Botryosphaeria* sp. When applied as foliar spray on grapevine plants growing in substrate artificially infested with *Neon. liriodendri* or *Pa. chlamydospora*, chitosan significantly reduced the percentage of isolation of both pathogens, enhancing the growth of the vine plants infected with the first pathogen. Although different application methods were used, the effects achieved by chitosan are comparable with those obtained with some of the most effective fungicides tested against these two pathogens. Results show that chitosan could provide an alternative or complement to conventional fungicides in integrated protection management of grapevine wood diseases and declines.

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