Chitosan Oligomers and Copper Sulfate Induce Grapevine Defense Reactions and Resistance to Gray Mold and Downy Mildew

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ABSTRACT

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Chitosan (CHN), a deacetylated derivative of chitin, was shown to be efficient in promoting plant defense reactions. CHN oligomers of different molecular weight (MW) and degree of acetylation (DA) triggered an accumulation of phytoalexins, *trans*- and *cis*-resveratrol and their derivatives ε-viniferin and piceid, in grapevine leaves. Highest phytoalexin production was achieved within 48 h of incubation with CHN at 200 μg/ml with an MW of 1,500 and a DA of 20% (CHN1.5/20), while oligomers with greater MW were less efficient, indicating that a specific MW threshold could be required for phytoalexin response. Treatment of grapevine leaves by highly active CHN1.5/20 also led to marked induction of chitinase and β-1,3-glucanase activities. CHN1.5/20 applied together with copper sulfate ($CuSO_4$) strongly induced phytoalexin accumulation. $CuSO_4$ alone, especially at low concentrations also elicited a substantial production of phytoalexins in grapevine leaves. Evidence is also provided that CHN1.5/20 significantly reduced the infection of grapevine leaves by *Botrytis cinerea* and *Plasmopara viticola*, and in combination with $CuSO_4$ conferred protection against both pathogens.

Additional keywords: elicitor, *Vitis vinifera*.

Plants can express defense mechanisms that provide protection against various pathogenic microorganisms. Successful protection depends on the timely recognition of signals or elicitor molecules generated or released during pathogen attack and the rapid induction of appropriate plant defense reactions (24,34). These defense responses include production of reactive oxygen species, phytoalexin biosynthesis, reinforcement of plant cell walls, and the accumulation of pathogenesis-related (PR) proteins, some of which possess antimicrobial properties (5,29,46,50). Similar defense responses can also occur upon treatment of plants with elicitor molecules before contact with the pathogen (7,9,28, 36,38,47).

Various types of elicitors have been reported in the literature, including oligosaccharides derived from the cell wall of various fungi, bacteria, and host plants (6,11,16,19,24,31), or from marine algae (7,31,36,38). In recent years, the importance of chitosaccharides as plant growth promoting and disease control agents has been emphasized (3,4,49,51). Chitosan (CHN), a beta-1,4-linked glucosamine, is a totally or partially deacetylated derivative of chitin (26). It has been shown to elicit a variety of defense reactions in higher plants such as the stimulation of phenylalanine ammonia lyase (PAL), peroxidase, and lipoxygenase activities, as well as the accumulation of phytoalexins and PR proteins (3, 15,22,27,31,49). CHN oligomers are also active elicitors of lignification in wounded and intact wheat leaves (8,51). Their biological activity was thought to result from their binding to membrane receptors (20) and to depend on the molecular weight (MW) and degree of *N*-acetylation of the molecule (DA) (35,51). In melon plants, CHN oligomers with a DA over 10% were shown to stimulate chitinase activity (45), and with a DA of less than 20% they also acted as an elicitor of PAL activity in wheat leaves (51). CHN oligomers of low MW were shown to be more effective in inducing defense responses than those of higher MW. Furthermore, some CHNs have been reported to enhance systemic resistance against pathogenic fungi in tomato (9) and rice seedlings (3).

Gray mold, caused by the fungus *Botrytis cinerea*, and downy mildew, caused by *Plasmopara viticola*, are among the most damaging diseases of grapevine (*Vitis vinifera* L.). Control of these pathogens is generally achieved with chemical fungicide and copper salt applications. However, the appearance of fungicide-resistant pathogen strains and negative environmental impacts associated with these practices has intensified the need for reducing chemical use and for alternative disease management methods. In grapevine, the most thoroughly characterized inducible defense reactions by pathogenic fungi are the accumulation of phytoalexins and the synthesis of PR proteins including chitinases and β-1,3-glucanases (10,13,17,21,33). The main phytoalexins, *trans*-resveratrol and viniferin, are considered to be fungitoxic at physiological concentrations against *B. cinerea* (2) and can enhance grapevine plant resistance against *P. viticola* (18,41). Similar responses have also been activated by inducers of systemic acquired resistance, wounding, aluminum chloride, jasmonates, and UV irradiation (12,13,48). Recently, Hamiduzzaman et al. (28) reported that β-aminobutyric acid also potentiated expression of PR protein genes and induced resistance of grapevine plants against *P. viticola*. Similarly, algal β-1,3-glucan laminarin and oligogalacturonides elicited a variety of defense responses in grapevine cell suspension cultures and leaves, including accumulation of phytoalexins and activation of a set of PR genes and proteins (6,7). In addition, the pretreatment of grapevine leaves or

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plants by these oligosaccharides conferred significant resistance against *B. cinerea* and *P. viticola*.

This study was undertaken to determine whether treatment of grapevine leaves with water-soluble CHN oligomers induces resistance to *B. cinerea* and *P. viticola*. For this purpose, the elicitor activity of CHN oligomers with a wide range of known MW and DA was investigated through phytoalexin accumulation in grapevine leaves. We further examined the ability of the most active CHN to induce chitinase and β-1,3-glucanase activities, and to protect grapevine leaves against *B. cinerea* and *P. viticola*. In addition, the combination of this CHN oligomer with $CuSO_4$ at low concentrations in inducing phytoalexin accumulation and resistance of grapevine leaves against both pathogens was also investigated.

MATERIALS AND METHODS

Biological materials. Grapevine plantlets (*Vitis vinifera* cv. Chardonnay 7535) were obtained by multiplication in vitro on modified Murashige and Skoog (40) medium, pH 5.9 (half concentration of macroelements and glutamine at 200 mg/liter), supplemented with sucrose (20 g/liter) and agar (7 g/liter). Plantlets were grown at 25°C with a photoperiod of 16 h of light. Grapevine plants were obtained from cuttings cultivated in the greenhouse (24 to 28°C).

B. cinerea strain 630 (obtained from Y. Brygoo, INRA, Versailles, France) was grown on potato dextrose agar at 22°C. *P. viticola*-infected leaves were harvested in a Champagne vineyard (France) and sporangia were washed from infected leaves by brushing them into sterile distilled water. They were either used freshly or stored in a sterile solution containing 10% glycerol at –80°C.

Chemicals. Crab-shell CHN oligomers with an MW of 1,500 to 10,000 and DA from 2 to 30% were obtained from Agrolor (Nancy, France). They were prepared according to the method of Domard and Cartier (23). Copper sulfate was purchased from Sigma (St. Louis, MO).

Treatments. Leaves were excised from 10-week-old grapevine in vitro grown-plantlets (cv. Chardonnay) and incubated by floating them on a reference buffer (2 mM MES, pH 5.9, containing 0.5 mM $CaCl_2$ and 0.5 mM K_2SO_4). The elicitation process was carried out with CHN fragments differing in their MW and DA at concentrations of ≤300 μg/ml. CHN was also used in combination with $CuSO_4$ at concentrations ranging from 1 to 100 μg/ml. Both CHN and copper sulfate were dissolved in the reference buffer adjusted to pH 5.9.

Phytoalexin quantification. Grapevine leaves were collected at different times after treatment and ground in liquid nitrogen with a chilled mortar and pestle, and 4 ml of 95% methanol was added to 1 g of the obtained powder. All extraction steps were done in subdued light conditions. After centrifugation (10 min, 5,000 × *g*), supernatants were evaporated under nitrogen and phytoalexins were solubilized by addition of 1 ml of 100% methanol. This solution was clarified by filtration through a Millex-GN 0.22-μm filter (Millipore, St-Quentin en y, France) before high-performance liquid chromatography analysis. Twenty microliters of each sample was loaded onto a Lichrocart C-18 reversed phase column (250 mm × 4 mm, 5 μm, Waters, St-Quentin en y, France) equilibrated with a 90:10 (vol/vol) H_2O/acetonitrile mobile phase. Phytoalexins were eluted with a linear gradient of 10 to 85% acetonitrile at a flow rate of 1 ml/min. *Trans*- and *cis*-phytoalexins were detected with a photodiode array detector coupled to a fluorometer detector (λex = 330 nm, λem = 374 nm) and quantified on the basis of a standard calibration curve of *trans*-resveratrol, as previously described (32,43).

Chitinase and β-1,3-glucanase activity assays. Enzymes were extracted by grinding 250 mg of fresh leaves in 2 ml of 50 mM sodium acetate buffer, pH 5, containing 1 mM dithiothreitol and 0.2% (wt/vol) phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 9,000 × *g* for 10 min at 4°C and supernatants were used for enzymatic activity assays. Chitinase and β-1,3-glucanase activities were assayed according to the method of Wirth and Wolf (52) using carboxymethyl-chitin-Remazol-Brilliant Violet (CM-chitin-RBV) and carboxymethyl-curdlan-Remazol-Brilliant Blue (CM-curdlan-RBB) as respective substrates (Loewe Biochemica, Germany).

Protection assays. For *B. cinerea* infections, conidia were collected with 10 ml of sterile water from a 10-day-old potato dextrose agar culture, filtered to remove mycelia, and counted. For each treatment, 30 leaves were excised from 10-week-old grapevine in vitro-grown plantlets and pre-incubated by floating them on a standard buffer (2 mM MES, 0.5 mM $CaCl_2$, and 0.5 mM K_2SO_4, pH 5.9) containing various concentrations of CHN (CHN1.5/20) and/or $CuSO_4$ at defined concentrations. After 48 h, leaves were rinsed with distilled water and placed on wet absorbing paper in plastic petri dishes. One needle-prick wound was applied to the abaxial surface of each leaf, and the fresh wounds were covered with 5-μl drops of a suspension of 5×10^5 conidia per ml. Quantification of disease development was measured as the average diameter of lesions formed 6 days postinoculation.

For *P. viticola* infections, cuttings with seven to eight expanded leaves were first sprayed on both leaf surfaces with solutions of CHN1.5/20 at 200 μg/ml with or without $CuSO_4$ at 50 μg/ml. Plants were placed in a growth chamber (25°C, 16 h of daylight, 70% relative humidity) for 8 days, and then the abaxial surface of detached leaves was inoculated by application of 20-μl drops of a suspension of 5×10^4 sporangia per ml. Inoculated leaves were placed in glass covered dishes, incubated for 16 h in the dark, and then placed in a growth chamber under the conditions described above. Disease was estimated 8 days postinoculation by measuring the percentage of leaf area covered with mycelia. Analysis of variance and Duncan's multiple range test were performed for all assays.

RESULTS

Time course of phytoalexin production in response to CHN. Resveratrol (*trans*-3,4′,5-trihydroxystilbene) and its dehydrodimer ε-viniferin have been reported as the major phytoalexins produced by grapes (*Vitis* spp.) in response to microbial infection and associated with resistance to fungal pathogens (2,17,33,42). Here grapevine leaves were challenged by two CHNs (200 μg/ml) differing in MW and DA; CHN1.5/20 with MW of 1,500 and a DA of 20%, and CHN10/2 with MW of 10,000 and a DA of 2%. As shown in Figure 1, induction of resveratrol and derivatives, *trans*- and *cis*-viniferin and piceid, was observed following treatment of grapevine leaves with CHNs. The accumulation of *trans*-resveratrol (Fig. 1A) increased more rapidly than its *cis* isomer (Fig. 1B) and their metabolites, *trans*- and *cis*-ε-viniferin (Fig. 1C and D) and piceid (Fig. 1A and F). For both CHNs, the production of phytoalexins showed similar profiles with maximum accumulation of resveratrol and ε-viniferin at 48-h postelicitation. Thereafter, *trans*-resveratrol accumulation declined slightly, while ε-viniferin and piceid accumulation remained stable until 72 h. Amounts of phytoalexins were generally greater with CHN1.5/20 (by two- to threefold) than with CHN10/2, suggesting that phytoalexin response could be dependent on either the MW or the DA of CHN.

Effects of MW and DA of CHN on phytoalexin production. In order to investigate whether MW and DA of CHN can influence phytoalexin accumulation in grapevine, leaves were treated for 48 h with different CHN of 1.5, 5, and 10 kDa and DA ranging from 2 to 30% at a concentration of 200 μg/ml. The accumulation of all elicited phytoalexins was directly dependent upon the MW of the CHN backbone (Fig. 2). The *trans* isomers remained pre-

dominant and were more influenced by the CHN structure than *cis* isomeric forms. The pattern of the resveratrol response (Fig. 2A) was similar to that of ε-viniferin (Fig. 2B) and piceid (Fig. 2C) in that, in all cases, the larger CHNs (10 kDa) were less active. The CHN with low molecular mass (1.5 to 3 kDa) induced the largest accumulation of all phytoalexins. The DA appeared to influence the elicitor activity of CHN. Phytoalexin production increased with decreasing DA, with some dependency on MW. The highest phytoalexin accumulation was obtained with a DA of 2 to 20% for CHN of 1.5 and 3 kDa. For both MW, the maximum production of resveratrol and ε-viniferin was observed with a DA of 20%. Maximum piceid levels (Fig. 2C) were also obtained with CHN of low MW and a DA of 20%, indicating that both MW and DA can modulate CHN elicitor activity.

Dose-response curves for phytoalexin production by CHN1.5/20. When grapevine leaves were incubated in the presence of CHN1.5/20 for 48 h, the production of *trans*- and *cis*-resveratrol and piceid (Fig. 3A) increased with increasing concentrations of CHN. ε-Viniferin (Fig. 3B) also accumulated under its *trans* form together with a small amount of the *cis* isomer. The accumulation of major phytoalexins did not increase with treatments of CHN greater than 200 µg/ml (Fig. 3), while *cis*-ε-viniferin content significantly increased at CHN concentrations higher than 200 µg/ml.

Chitinase and β-1,3-glucanase activities in response to CHN1.5/20. Chitinase and β-1,3-glucanase are PR proteins that are considered to have antimicrobial activity against different pathogens. In grapevine leaves, the most active CHN in terms of phytoalexin production induced chitinase and β-1,3-glucanase activities (Fig. 4) with similar trends; both enzyme activities were increased at 10 h after CHN treatment with maximum accumulation at 24 h, and activity remained high for at least 48 h. Dose-response experiments showed that both PR enzymes are clearly stimulated by a CHN concentration of 100 µg/ml. Maximal induction of chitinase and β-1,3-glucanase activities was obtained with a concentration of 200 to 300 µg/ml. β-1,3-Glucanase activity was about fourfold greater than chitinase activity in response to CHN1.5/20.

Phytoalexin accumulation in response to CHN1.5/20 and copper sulfate. In order to optimize grapevine defense reactions and disease control with elicitor treatments, the highly active CHN, CHN1.5/20, was used at 200 µg/ml in combination with low doses of $CuSO_4$ ranging from 1 to 100 µg/ml. As expected, CHN1.5/20 alone induced accumulation of phytoalexins in leaves, especially the *trans* isomeric forms (Fig. 5). When leaves were incubated with $CuSO_4$ alone for 48 h, the production of *trans*- and *cis*-resveratrol (Fig. 5A and B), *trans*- and *cis*-ε-viniferin (Fig. 5C and D), and *trans*- and *cis*-piceid (Fig. 5E and F) increased with increasing concentrations of $CuSO_4$, reaching a maximal amount at 50 µg/ml $CuSO_4$ for *trans* isomers and from 5 to 50 µg/ml $CuSO_4$ for the *cis* isomers. The *trans* isomers remained predominant and were more influenced by the $CuSO_4$ concentration than the *cis* isomers. At a $CuSO_4$ concentration of 100 µg/ml, the amount of all forms of phytoalexins declined. The combination

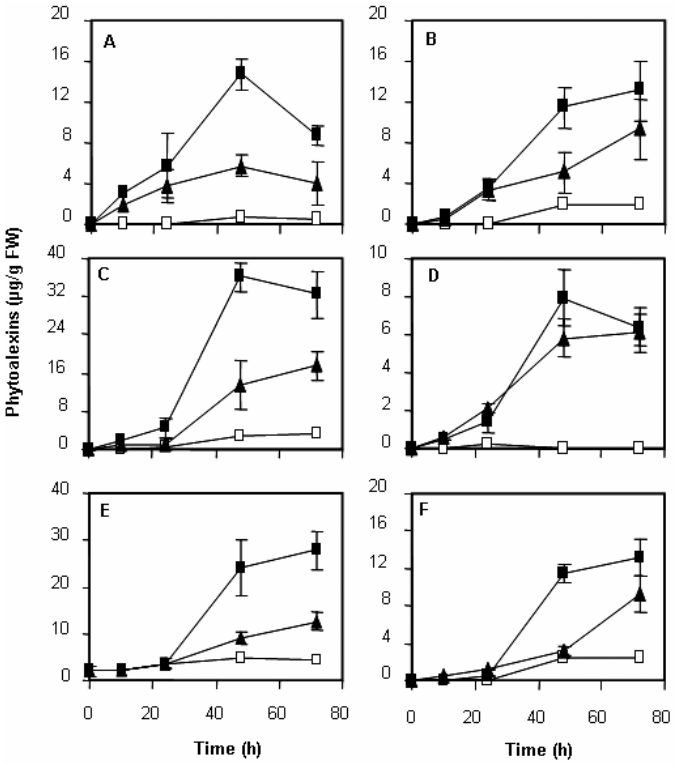

Fig. 1. Time-response curves of phytoalexin elicitation in grapevine leaves by chitosan (200 µg/ml) of different molecular weight and degree of acetylation (DA). CHN1.5/20, 1.5 kDa and DA 20% (solid square); CHN10/2, 10 kDa and DA 2% (solid triangle); and buffer control (open square). Data are means ± standard deviation from three independent experiments. **A,** *trans*-resveratrol; **B,** *cis*-resveratrol; **C,** *trans*-ε-viniferin; **D,** *cis*-ε-viniferin; **E,** *trans*-piceid; and **F,** *cis*-piceid.

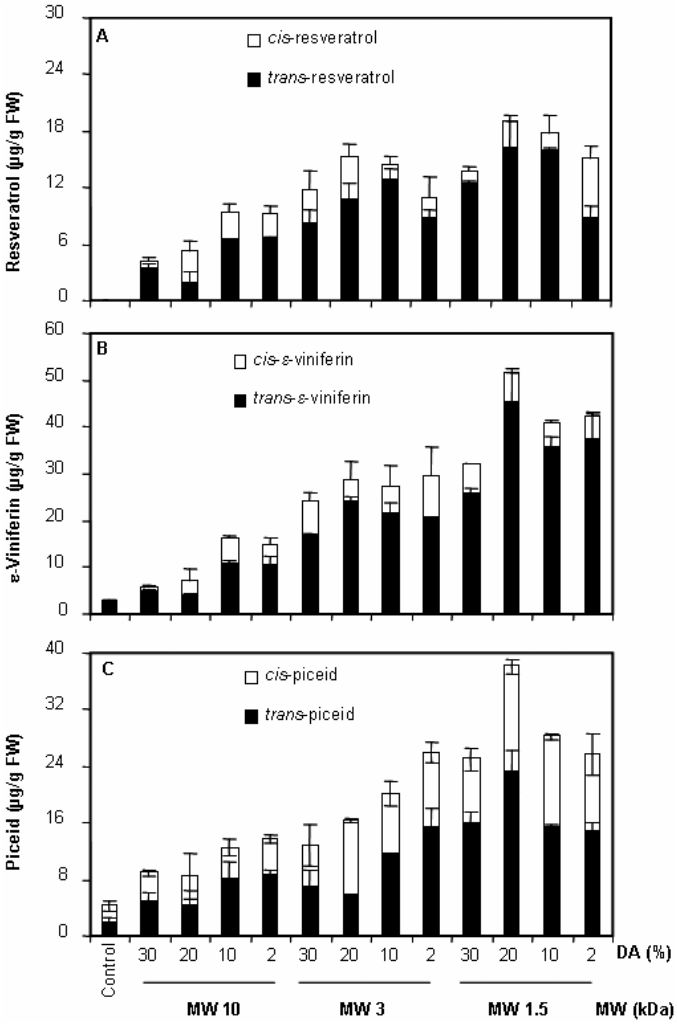

Fig. 2. Influence of the molecular weight and degree of acetylation on the activity of chitosan oligomers as elicitors of phytoalexins in grapevine leaves. Chitosan oligomers were applied at a final concentration of 200 µg/ml and leaves were harvested at 48 h for analysis of phytoalexins. Data are means ± standard deviation from three independent experiments. Each bar represents the sum of *trans*- (solid part of the bar) and *cis*- (open part of the bar) phytoalexin. **A,** *trans*- and *cis*-resveratrol; **B,** *trans*- and *cis*-ε-viniferin; and **C,** *trans*- and *cis*-piceid.

of both CHN and copper sulfate at concentrations ≤10 µg/ml was not significantly more effective than CHN alone for eliciting phytoalexins, but resulted in a strong accumulation of *cis*-resveratrol and *cis*-ε-viniferin when copper concentration exceeded 10 µg/ml.

Symptoms induced in grapevine leaves. Grapevine leaves incubated with CHN at concentrations ≤300 µg/ml did not develop any symptoms. However, treatment with $CuSO_4$ alone at concentrations of 50 to 100 µg/ml resulted in small necrotic spots after 2 days. Incubation of leaves with both CHN and $CuSO_4$ did not result in any disease symptom (data not shown).

Induction of resistance to *B. cinerea* and *P. viticola*. Because CHN1.5/20 induced different defense responses that reached their maximum within 48 h, we tested whether CHN would induce resistance against pathogen infection. Grapevine leaves excised from plantlets were pre-incubated with different concentrations of CHN, or buffer for 48 h. Treated leaves were challenge-inoculated with the gray mold agent (*B. cinerea*). Figure 6 shows the diameter of lesions (symptoms) 6 days postinoculation. CHN induced significant protection against gray mold infection depending on its concentration. The average lesion diameter was reduced by about 50% in the 50 µg/ml CHN-treated leaves (Fig. 6A). With CHN at 100, 200, and 300 µg/ml, this reduction level reached 60%. In the presence of $CuSO_4$, lesions were significantly smaller than those occurring in leaves treated with only CHN (Fig. 6B). Similar effects were observed when CHN and $CuSO_4$ were sprayed on the leaves (data not shown). Treatment with CHN1.5/20 reduced the infection to a greater extent than treatment with CHN3/30 or CHN10/20 (data not shown). A similar degree of resistance was observed when *B. cinerea* was inoculated 4 days after CHN treatment (data not shown).

CHN-triggered resistance against *P. viticola* was measured in assays in which leaves from potted plants were sprayed on both

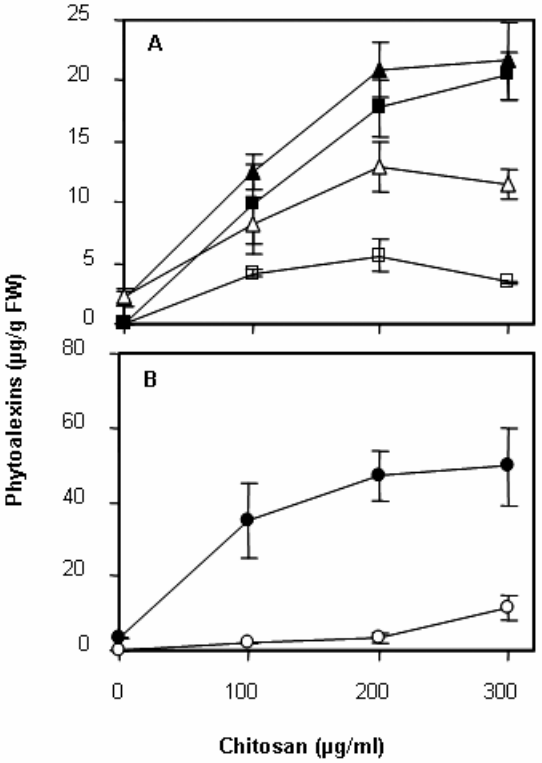

Fig. 3. Dose-dependent production of phytoalexins in grapevine leaves treated with chitosan of 1.5 kDa and degree of acetylation of 20%. Leaves were harvested at 48 h for analysis of phytoalexins. Data are means ± standard deviation from three independent experiments. **A,** *trans*- (solid square) and *cis*-resveratrol (open square), *trans*- (solid triangle), and *cis*-piceid (open triangle); and **B,** *trans*- (solid circle) and *cis*-ε-viniferin (open circle).

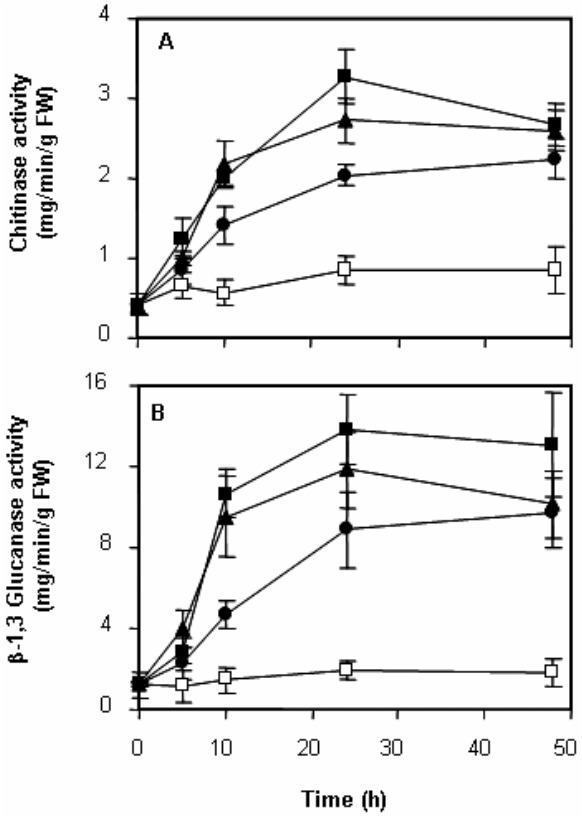

Fig. 4. Time-course and dose-response curves of **A,** chitinase and **B,** β-1,3-glucanase activities in grapevine leaves treated with chitosan of 1.5 kDa and degree of acetylation of 20%. Data are means ± standard deviation from three independent experiments. Control (0 µg/ml, open square), 100 µg/ml (solid circle), 200 µg/ml (solid triangle), and 300 µg/ml (solid square).

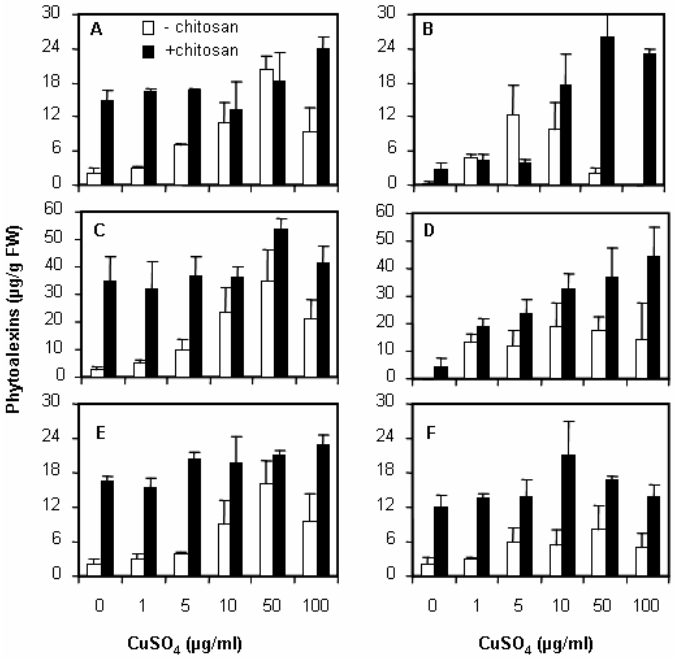

Fig. 5. Changes in the phytoalexin content of grapevine leaves treated with CHN1.5/20 and copper. Copper ($CuSO_4$) was applied at concentrations ranging from 0 to 100 µg/ml alone (open bars) or in the presence of CHN1.5/20 at 200 µg/ml (solid bars). Leaves were harvested at 48 h for analysis of phytoalexins. Data are means ± standard deviation from three independent experiments. **A,** *trans*-resveratrol; **B,** *cis*-resveratrol; **C,** *trans*-ε-viniferin; **D,** *cis*-ε-viniferin; **E,** *trans*-piceid; and **F,** *cis*-piceid.

surfaces with CHN at 200 µg/ml with or without $CuSO_4$ at 50 µg/ml. After 8 days, the time needed for maximum induction of several defense reactions (data not shown), an aliquot of sporangial suspension was inoculated to the abaxial surface of leaves. Disease intensity was estimated 8 days postinoculation by measuring the leaf area covered by the oomycete. Results (Fig. 6C) indicate that CHN reduced the percentage of *P. viticola*-infected leaf surface from 42% in the control to 12% in the CHN-sprayed leaves, corresponding to a 71% reduction in infection. Foliar sprays of a mixture of CHN at 200 µg/ml and $CuSO_4$ at 50 µg/ml reduced pathogen growth by 85%. $CuSO_4$ alone partially protected grapevine leaves against *P. viticola* by about 38%.

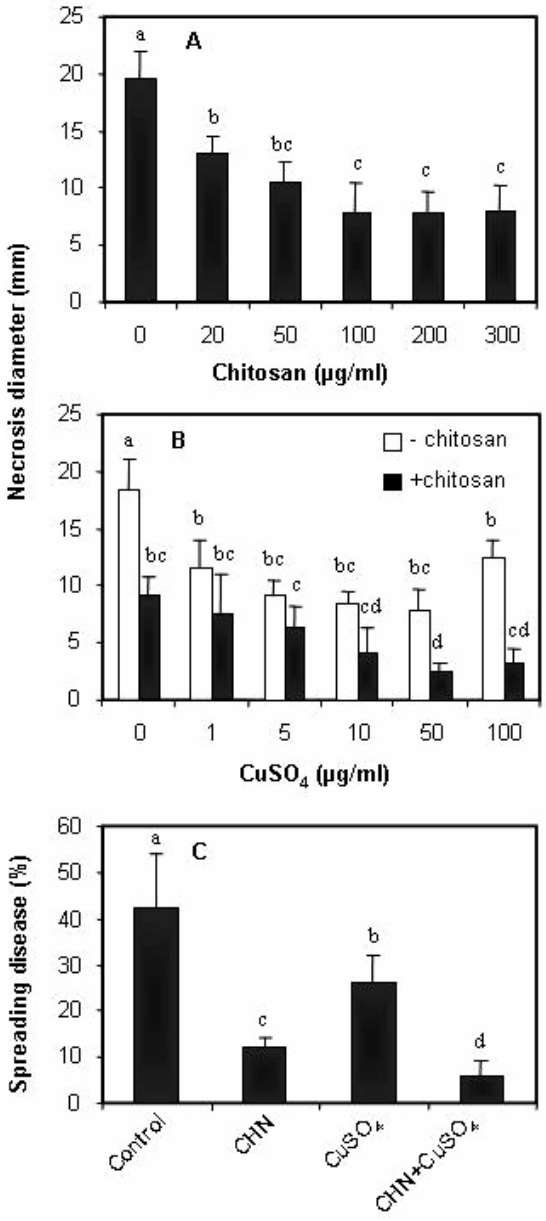

Fig. 6. Chitosan and copper sulfate-induced protection of grapevine leaves against *Botrytis cinerea* and *Plasmopara viticola*. **A,** Excised leaves were pretreated with CHN1.5/20 at concentrations ranging from 0 to 300 µg/ml for 48 h before challenge with *B. cinerea*. **B,** Excised leaves were pretreated with $CuSO_4$ alone (open bars) at concentrations ranging from 0 to 100 µg/ml or together with CHN1.5/20 at 200 µg/ml (solid bars) for 48 h and then drop-inoculated with *B. cinerea*. Data points are means ± standard deviation of lesions formed 6 days postinoculation on 30 leaves. Values with the same letter are not significantly different ($P \leq 0.05$). **C,** Development of downy mildew (*Plasmopara viticola*) on grapevine leaves sprayed with CHN1.5/20 at 200 µg/ml and/or $CuSO_4$ at 50 µg/ml. Data are means ± standard deviation of infected leaf surface from 10 to12 different leaves per treatment. Values with the same letter are not significantly different ($P \leq 0.05$).

Effects of CHN and $CuSO_4$ on the pathogens. The direct effects of CHN with or without $CuSO_4$ on *B. cinerea* and *P. viticola* were also investigated. The anti-*Botrytis* activity was determined by measuring the mycelium growth on potato dextrose agar medium (17) supplemented with CHN (50 to 300 µg/ml) at pH 5.9. After 5 days of incubation, CHN concentration below 200 µg/ml did not significantly inhibit *B. cinerea* growth. The highest CHN concentration caused only 10 to 15% inhibition of fungal growth. The combination of CHN at 200 µg/ml with $CuSO_4$ at 50 µg/ml also resulted in a similar effect. This antifungal activity tended to decrease with time (data not shown). For *P. viticola*, the direct effect was assayed on fresh grapevine leaves sprayed with CHN at 200 µg/ml alone or together with $CuSO_4$ at 50 µg/ml and immediately inoculated with sporangia. In both cases, pathogen growth was only slightly inhibited (by about 15 to 20%).

DISCUSSION

CHN (β-1-4 linked *N*-glucosamine) has been shown to induce defense responses in different plants (15,25,27,51). The data reported here show that CHN oligomers also induce defense responses in grapevine leaves, as evidenced by an accumulation of stilbene phytoalexins, *trans*- and *cis*-resveratrol, ε-viniferins, and piceids, and a stimulation of chitinase and β-1,3-glucanase activities. Furthermore, the combination of CHN and $CuSO_4$ increased phytoalexin production. This elicitor capacity of CHN and/or $CuSO_4$ appeared to be associated with an induced protection of grapevine leaves against gray mold and downy mildew diseases.

Accumulation of *trans*-resveratrol, a reaction product of stilbene synthase, has been considered the primary inducible response of grapevine against a number of biotic and abiotic stresses (17,33,43). The low amounts of *cis*-resveratrol recovered could result from a photoisomerization of its *trans* isomer, or from breakdown of the glucoside *cis*-piceid. The production of ε-viniferin and piceid suggests that CHN can induce a rapid dimerization and glycosylation of resveratrol, respectively. Alternatively, ε-viniferin could also be produced through an oxidative coupling of resveratrol catalysed by a cell wall-localized peroxidase (14). These processes might be distinguished by comparing kinetics of accumulation of both *trans*-resveratrol and their metabolites in response to CHN. The induction of resveratrol and ε-viniferin production by CHN is consistent with the induced PAL activity in grapevine leaves by CHN (49). Increasing the synthesis of stilbenes would be expected to enhance the plant's defense capacity. Most of these compounds have been shown to exhibit rather unspecific antimicrobial properties at physiological concentrations, enhancing the resistance of grapevine plants to *B. cinerea*, *Plasmopara viticola*, and *Phomopsis viticola* (18,30,39,41).

The amount of all elicited phytoalexins was directly dependent upon MW and DA of CHN (Fig. 2). The *trans* isomers remained predominant and were more influenced by the CHN structure than *cis* isomeric forms. In all cases, CHN with a minimum chain length (1.5 kDa) and a DA of ≤20% exhibited a high phytoalexin-inducing activity. The highly active CHN (CHN1.5/20) also induced chitinase and β-1,3-glucanase activities to a greater extent than laminarin (7) or oligogalacturonides (6). Both PR proteins are considered to be functionally implicated in the defense response directed toward chitin and β-glucans, which are major cell wall components of various fungi (50). These data corroborated some earlier findings showing that CHN oligomers ranging from 1.1 to 1.34 kDa and DA of <20% were more active than CHN of higher MW in inducing PAL activity in wheat (51) and phytoalexin production in rice leaves (53). Our results also underline the importance of the acetylation process for CHN biological activity. CHNs with lower DA had greater activity, suggesting that

the chitin portion present in the CHN could be responsible for induced plant defense responses, through binding to a receptor on the plasma membrane (20). It has also been proposed that the activity of CHN with a low DA could be attributed to the charges along the chitin backbone resulting in the formation of multi-oligomer complexes with membrane components (35).

Treatments of $CuSO_4$ alone or in combination with CHN induced accumulation of resveratrol and their metabolites in grapevine leaves. Interestingly, the induction of *cis*-resveratrol and *cis*-ε-viniferin was very responsive to $CuSO_4$, especially when its concentration exceeded 10 μg/ml. To our knowledge, this is the first report showing that $CuSO_4$ enhanced the elicitor-induced defense reactions in plants. Some of these results are in agreement with those of Rakwal et al. (44) and Adrian et al. (1) who showed that copper chloride and aluminium chloride elicited phytoalexin production in rice and grapevine leaves, respectively. The strong accumulation of *cis*-resveratrol and *cis*-ε-viniferin could be explained by an enhanced release of *cis*-resveratrol from the glucoside *cis*-piceid. These compounds can be considered important markers for induced resistance of grapevine to the pathogens. The oxidative dimerization of resveratrol has been reported to be associated to resistance of grapevine cultivars to *P. viticola*, whereas its glycosylation is associated to sensitivity to this pathogen (42). In our system, $CuSO_4$ alone (50 to 100 μg/ml) provoked slight necroses in grapevine leaves after 2 days, while CHN (up to 300 μg/ml) did not induce any symptom even in the presence of $CuSO_4$ (data not shown). This indicates that unlike $CuSO_4$, the CHN-eliciting activity is not associated with cell death in grapevine leaves and that CHN can prevent possible $CuSO_4$-induced cell death (37). CHN can act as a chelating agent that binds $CuSO_4$. Moreover, one cannot exclude that sulfate groups might play a critical role in the $CuSO_4$/CHN-induced defense responses in grapevine. Recent reports (36,38) have shown that sulfated oligosaccharides were more active than the unsulfated molecules in triggering various defense responses and systemic resistance against microbial pathogens.

The defense responses induced by CHN correlated with a reduction of *B. cinerea* and *P. viticola* infection (by about 60 and 70%, respectively). The combination of CHN with copper sulfate enhanced both phytoalexin accumulation and protection against the pathogens. Several of these effects were checked and confirmed in leaves treated by floating or spraying them with CHN and $CuSO_4$ (data not shown). The induced protection did not apparently result from any direct effect on pathogen growth but rather from greater induced plant resistance. Application of CHN1.5/20 at concentrations up to 300 μg/ml with or without $CuSO_4$ (50 μg/ml) to culture media or leaf surfaces just before inoculation only resulted in a slight growth inhibition of *B. cinerea* and *P. viticola*. These data are in agreement with other findings showing that increased resistance to *B. cinerea* and *P. viticola* was obtained in grapevine plants with high phytoalexin and PR protein levels (12,13,17,42).

Taken together, our results demonstrate that CHN induces an accumulation of stilbene phytoalexins in grapevine leaves depending on its concentration, MW, and DA. The most active CHN oligomer also activated PR proteins in grapevine leaves. In addition, a combination of this CHN with low doses of $CuSO_4$ resulted in a substantial accumulation of phytoalexins. This elicitor capacity of CHN and $CuSO_4$ appeared to be associated with an induced protection against gray mold and downy mildew diseases, which resulted rather from greater induced plant resistance than from direct effect on the pathogens.

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