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Chitosan induces delayed grapevine defense mechanisms and protects grapevine against *Botrytis cinerea*

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Abstract

In the present study, a commercial chitosan soluble in acid solution and obtained from shrimp shell waste, with a molecular weight of 173 kDa and a degree of acetylation of 17%, named as chitosan (173/17), was investigated. Chitosan is a well-known biopolymer whose antimicrobial properties are highly influenced by the molecular weight, degree of acetylation as well as the preparation and derivatization methods used. Chitosan (173/17) was applied on grapevine leaves before *Botrytis cinerea* inoculation to verify its effectiveness as a preventive treatment against the fungal infection. The expression of a set of defense marker genes, as well as accumulation of stilbene phytoalexins, was investigated. Thanks to its fungistatic and filmogenic properties, chitosan (173/17) protected grapevine leaves against *B. cinerea.* Moreover, it induced grapevine defense response: three days after the treatment an induction of the jasmonic acid and ethylene-mediated response, a repression of the salicylic acid-mediated signaling, and a transient accumulation of *trans*-resveratrol were registered. Our data indicate that chitosan (173/17), when used in preventive application, is able to protect grapevine against *B. cinerea* infection. The effectiveness of chitosan (173/17) as a natural ecofriendly product for the control of *B. cinerea* on grapevine was demonstrated.

Keywords *Botrytis cinerea* · Chitosan · Defense response · Gene expression · Grapevine

Introduction

Grapevine (*Vitis vinifera* L.), one of the most economically important fruit species grown around the world, is susceptible to many fungal pathogens, such as *Botrytis cinerea*, which induces gray mold disease at various developmental stages and on different plant organs (Mohamed et al. 2007). Generally, the disease is controlled through spraying of phytochemicals. On the other hand, due to negative impact of phytochemicals on environment, on quality of food and beverage products, and on human health, there is a strong demand from the society for alternatives based on natural methods of disease control.

Several alternatives to fungicides have been reported in the literature against grapevine *B. cinerea*, such as seaweed (Jeandet et al. 2000), laminarin (Aziz et al. 2003), oligogalacturonides (Aziz et al. 2004), plant extract of *Reynoutria sachalinensis* (Elmer and Reglinski 2006), rhamnolipids (Varnier et al. 2009), *Saccharomyces* (Pujos et al. 2014), and chitosan (Ait Barka et al. 2004; Aziz et al. 2006; Trotel-Aziz et al. 2006; Reglinski et al. 2010).

Chitosan is a natural non-toxic polymer of β-1,4-linked glucosamine obtained by deacetylation of chitin, which can be obtained from shrimps and crabs (crustaceans shell), mollusks (endoskeleton of cephalopods), fungi and algae cell walls and insects (exoskeleton). However, commercial chitosan is mainly recovered from marine sources waste, i.e., the crustaceans processing industries. Indeed, more than 10,000 tons of chitosan could be available every year from shellfish waste (Merzendorfer 2009).

Its biological activity is related to the size of the polymer and to the degree of acetylation (DA) (Kauss et al. 1989;

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Hadwiger et al. 1994). The in vitro activity of formulations containing putative low molecular weight chitosan (LMW), but without any specification on the exact molecular weight (MW), as Armour-Zen® and Chitogel®, has been reported on grapevine plantlets and detached leaves against *B. cinerea* (Ait Barka et al. 2004; Reglinski et al. 2010). Aziz et al. (2006) and Trotel-Aziz et al. (2006) used chitosan with 1.5–10 kDa and 5 kDa MW, respectively, and both demonstrated an increase in defense-related enzyme activity and induced resistance to infection by *B. cinerea*. They showed that chitosan elicits a variety of defense reactions in plants such as the stimulation of phenylalanine ammonia lyase (PAL), peroxidase, and lipoxygenase activities, as well as the accumulation of phytoalexins and pathogenesis-related (PR) proteins. Comparing samples of chitosan with different MW, Trotel-Aziz et al. (2006) showed also that the induction activity decreased with the polymer dimension, moving from 1.5 to 10 kDa. Regarding the direct antifungal effect of chitosan, these authors found contrasting results on *B. cinerea*, with only small inhibitory effect until 0.3 g/L in one case (Aziz et al. 2006) and a maximum inhibitory effect at 0.15 g/L in the other case (Trotel-Aziz et al. 2006). The deacetylation degree was similar in the two experiments, so this parameter could not be responsible for such a difference.

Few data are available on the use of chitosan with higher MW. In this case, it should be considered that the increase in the polymer dimension affects also its solubility: chitosan with molecular weight below 20 kDa is still water-soluble (Cai-qin et al. 2002), while higher MW polymers need diluted acids to be dissolved, giving viscous solutions. This aspect can constitute a difficulty when formulating commercial preparations to be applied on plants but, on the other hand, the film-forming ability of viscous chitosan solutions could increase the protection effect against fungi by creating a physical barrier, as suggested by Ait Barka et al. (2004). Bhaskara Reddy et al. (2000) using a HCl-solubilized chitosan found a protective effect against *B. cinerea* in strawberry, hypothesizing a direct fungistatic effect and suggesting (but without demonstrating) even an effect on the induction of defense response. Hernández-Muñoz et al. (2006) demonstrated the ability of HCl-solubilized chitosan to inhibit the in vitro growth of *B. cinerea*, finding an IC_{50} (half maximal inhibitory concentration) of 1.77 g/L.

In the present study, a commercial chitosan solubilized in acid solution, obtained from shrimp shell waste, was chemically characterized, and its antifungal activity against *B. cinerea* infections was investigated by conducting in vitro assays (conidia germination and mobility and mycelium growth).

Furthermore, it was investigated whether the activation of plant defense mechanisms is related to the demonstrated ability of chitosan in protecting leaves from *B. cinerea* infection.

Material and methods

Chemical characterization of chitosan

The commercial chitosan from shrimp shell was purchased from Qingdao Yunzhou Biochemistry Co., Ltd (Jimo, Qingdao, China).

The degree of deacetylation was determined in triplicate according to the procedure described by Tolaimate et al. (2000) with some modifications. The chitosan (0.2 g) was dissolved in 20 mL of 0.1 M HCl solution and 25 mL of deionized water. After 30 min of continuous stirring, a second portion of 25 mL of deionized water was added and stirring continued for other 30 min. When chitosan was completely dissolved, the solution was titrated with NaOH 0.1 M solution using an automatic titrator (Hanna Instruments, model HI 901, Woonsocket, RI, USA), and a curve with two inflexion points was obtained. The difference of the volumes of these two points corresponds to the acid consumed for the salification of amine groups that was used to determine the degree of acetylation of the chitosan.

The intrinsic viscosity of chitosan was determined according to the methodology of Mao et al. (2004). The chitosan (0.050 g) was dissolved in 100 mL of 2% HAc/0.2 M NaAc, and the viscosity was measured in triplicate using an Ubbelohde glass capillary viscometer, with a viscosity range from 2.000 to 10.000 cSt (Fungilab, ASTM size 4, Sant Feliu del Llobregat, Barcelona) in a constant temperature water bath at 25 ± 0.01 ºC. The capillary diameter used was 0.63 mm. Solution concentrations were adjusted based on the viscosity of the samples and the flow through time was kept in the range of 100–150 s. Five different concentrations were tested, and the calculation of intrinsic viscosity was obtained by common intercept of both Huggins and Kraemer plots. The value of intrinsic viscosity was used to estimate the molecular weight through the Mark–Houwink–Sakurada equation as described by Kasaai (2007).

Plant material

Grapevine plantlets of *V. vinifera* L. cv. Merlot clone Ampelos TEA 20 base category, a variety susceptible to *B. cinerea*, were placed in individual pots and grown in a glasshouse at a temperature of 24 and 18 ºC (day and night, respectively) with a 12-h photoperiod at 60 µmol m^{-2} s^{-1} cool-white light and $70 \pm 10\%$ relative humidity until they developed twenty leaves.

In vitro production of Botrytis cinerea inoculum

Botrytis cinerea was isolated from *Brassica oleracea* (provided by Simone Ferrari, Sapienza University of Rome, Italy) and cultured as described by Ferrari et al. (2003). The fungi were grown and maintained on malt extract agar (Amresco, Solon, Ohio, USA) in 90-mm Petri dishes at 21 °C under a photoperiod of 12 h. *B. cinerea* inoculum were harvested from 15- to 20-day-old cultures and collected by rubbing the plates with a glass rod with 10 mL of sterile distilled water. After filtration on sterile gauze to remove mycelia, conidia were pelleted by centrifugation at $5000 \times g$ (5 min) and further resuspended in 5 mL of sterile distilled water at a final concentration of 1×10^6 conidia/mL (Malassez chamber was used for conidia counting).

Analysis of antifungal properties of chitosan solution using two different systems

The antifungal properties of the chitosan solution were investigated both on nutrient agar medium and on grapevine (detached leaves and whole plants).

In vitro antifungal assays were carried out by adding different chitosan concentrations in 90-mm Petri dishes containing potato dextrose agar (PDA).

In detail, different volumes from a chitosan stock solution 30 g/L dissolved in 0.5% acetic acid were added to PDA medium before its solidification obtaining final chitosan concentrations of 0.5, 1, 2 and 3 g/L. For each condition (except for 3 g/L concentration), appropriate volumes of 0.5% acetic acid were added in order to have in all experiments the same final concentration of the organic acid. Two controls, with the addition of only water or 0.5% acetic acid, were performed. At least four plates were prepared for each condition. *B. cinerea* mycelium was inoculated on the center of the plate and maintained at 21 °C ± 1 °C under a photoperiod of 12 h. The mycelial development was observed until full plate coverage, and the measured mycelium area was compared with the PDA controls.

For assays performed on grapevine leaves (in vitro), fully expanded leaves, with on average 30 mm size long, were excised from plants (12–16 weeks old) and washed with water, surface-sterilized by immersion in 3% sodium hypochlorite for 4 min, and then rinsed twice in distilled water for 1 min (Danti et al. 2002). Then, four or five leaves were placed in Petri dishes (160 mm of diameter), the adaxial side facing wet adsorbent paper (Whatman). After a preliminary trial to identify the most suitable chitosan concentration, treatments were performed by spraying leaves with a solution of 1 g/L chitosan in 0.5% acetic acid, and after 24, 72, and 120 h, leaves were infected with *B. cinerea*. Depending on the size of the leaves, one-to-four lesions were applied to each leaf, and the fresh wounds were covered with 5 μL drops of the suspension of 1×10^6 conidia/mL in potato dextrose broth (PDB) (Carlo Erba, Rodano, Milan, Italy). The trial included seven experimental conditions (three plates for each condition): healthy (neither infected nor treated) leaves (named mock); leaves treated with chitosan and inoculated with *B. cinerea* at three different times (24, 72, and 120 h post-treatment) (named: Chit + Bc_24, Chit + Bc_72, Chit + Bc_120), and the respective untreated controls infected with *B. cinerea* at the same time (named: Bc_24, Bc_72, Bc_120). The plates were incubated at 24 ℃ ± 1 °C with a 12-h photoperiod. Humidity was maintained by covering the plates with a transparent plastic lid.

For whole plant assays (in vivo), plants were treated with 1 g/L chitosan in 0.5% acetic acid, and 24 h after treatment, twenty leaves per plants were inoculated with 10 μL drops of the conidial suspension in PDB (1×10^6 conidia/mL). Each plant was covered with a transparent plastic bag and incubated at 24 ℃ ± 1 °C with a 12-h photoperiod. The experimental design included four experimental conditions (three plants for each condition): healthy (neither infected nor treated) plants (mock); plants inoculated with *B. cinerea* (Bc); plants treated with chitosan (Chit); plants treated with chitosan and then inoculated with *B. cinerea* 24 h after chitosan treatment (Chit + Bc).

Fungal development was measured as average diameter of lesions formed from one to ten days post-infection (dpi) for both experiments on grapevine (detached leaves and whole plants).

Gene expression studies: sampling, RNA extraction and quantitative RT-qPCR

To understand whether treatment with the chitosan solution induced the activation of defense mechanisms, the expression of selected defense-related genes was investigated on uninfected leaves (treated and untreated) collected during in vivo assay (experimental conditions named: mock and Chit). Leaves were collected from at 1, 3, 6, and 9 days after treatment (dpt). Three independent biological replicates were collected from each experimental condition and each sampling time point, by excising 10-mm disks from four different leaves of each plant, for a total of 24 samples. After the collection, the leaves were immediately frozen in liquid nitrogen and stored at − 80 ℃.

Samples (100 mg) taken from frozen leaves were homogenized in liquid nitrogen, and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) with a protocol previously described by MacKenzie et al. (1997). One μg of RNA was treated with 1 U of RNase-free DNase I (Invitrogen) for 45 min at 37 ℃, and the reaction was stopped with 1 μl of 25 mM EDTA. After denaturation at 95 ℃ for 5 min, RNA was reverse-transcribed at 42 ℃ for 50 min with Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) and

DNA random primers (Roche Diagnostic) (Bertazzon et al. 2012).

Real-time PCR assays were carried out on a Bio-Rad thermal cycler (model CFX 96) in 96-well plates using the 2×Platinum SYBR Green qPCR Supermix UDG (Invitrogen). PCRs were performed in duplicate, in a total volume of 10 μL, including 0.3 mM of each primer and 1 μL of cDNA. The thermal protocol included a decontamination step of 3 min at 50 ℃ to allow for optimal UDG (Uracil DNA Glycosylase) enzymatic activity, followed by a step of 3 min at 95 ℃ in order to activate the Platinum Taq polymerase, to deactivate the UDG and to denature the DNA sample. Subsequently, 50 cycles of a two-step protocol, consisting of 5 s of denaturation at 95 ℃ followed by 30 s of annealing/extension at 60 ℃, were performed. Identical thermal cycling conditions were used for all the targets.

For the selection of reference genes, a set of five *V. vinifera* candidate reference genes (*actin, cytochrome oxidase, pyruvate decarboxylase, glyceraldehyde-3-phosphate dehydrogenase* and *26S rRNA*) was tested in the experimental conditions (supplementary table 1). The qbasePLUS software (Biogazelle) was used identifying *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *cytochrome oxidase* (*COX*) as the two most stably expressed genes.

The expression of some defense and stress-related genes was monitored. Several genes involved in the hormone-mediated signaling were considered: *Non-expressor of Pathogenesis-Related 1* (*NPR1*) and *Enhanced disease susceptibility 1* (*EDS1*), which are important components of the salicylic acid signaling; genes encoding Jasmonate ZIM domain proteins (*JAZ1.1*) and 13-lipoxygenase (*LOX13*) involved in the jasmonic acid-mediated signaling; *1-aminocyclopropane-1-carboxylate oxidase 1-like* (*ACO*) involved in ethylene biosynthesis and 9-lipoxygenase (*LOX9*) component of the oxylipin pathway. Moreover, *glutathione-S-transferase* (*GST1*), a gene involved in redox status, and the transcription factor *WRKY1* were analyzed. The expression level of five genes coding for PR protein was evaluated: *PR1, PR5* (*Thaumatin-like/Osmotin*), *PR6* (*Proteinase inhibitor*), *PR10* (*Ribonuclease-like*) and *PR14* (*Lipid transfer protein*). Finally, two genes were selected from the phenylpropanoid pathway: *PAL (phenylalanine ammonia-lyase)* and *STS1* (*stilbenes synthase 1*), both responsible for phytoalexin synthesis. The gene-specific primers were selected from the literature (supplementary Table 1). Transcript levels were calculated with the comparative Ct ($2-\Delta\Delta Ct$) method, all the genes showing similar amplification efficiencies, ranging between 90 and 100%.

Phytoalexin extraction and characterization by HPLC analysis

To investigate whether chitosan treatment induced the production of phytoalexins, the level of stilbene compounds was investigated on the same 24 samples collected for transcriptomic assays.

The stilbenoids extraction was performed according to the procedures described by Repetto et al. (2012) with some modifications. An amount of 200 mg of frozen leaves were ground in a mortar with liquid nitrogen and then extracted using 10 mL of methanol with 100 μL-of trans-4-idrossistilbene (internal standard). The extract was paper-filtered and evaporated under vacuum at 35 ℃ and the residue dissolved in methanol and water (1:1 v/v). The sample was then filtered with a 0.2 μm membrane and stored at−20 °C for quantification by HPLC.

The analysis of stilbenes was performed according to the procedure described by Vincenzi et al. (2013) with some modifications. Stilbenes were separated on a C18 Lichrospher column (4 mm×250 mm, 5 μm, Agilent Technologies, Milano, Italy) at 40 °C, using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector Waters 2487 (Waters Corporation, Milford, MA, USA). The mobile phase gradient was 0.5% v/v formic acid in deionized water (solvent A) and 2% v/v formic acid in methanol (solvent B). The gradient program was 0 to 10% (solvent B) in 3 min, followed by 10 to 30% (solvent B) in 5 min, 30 to 44% (solvent B) in 35 min, 44 to 55% (solvent B) in 2 min, 55 to 75% (solvent B) in 15 min and 75 to 100% (solvent B) in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate was 1.0 mL/min and the injection volume of 20 μL. Detection was performed at 306 nm for *trans*-isomers of piceatannol, resveratrol, and ε-viniferin. The concentration of individual stilbenes was quantified on the basis of peak areas using calibration curves of commercially available standards of *trans*-piceatannol, *trans*-resveratrol, and *trans*-ε-viniferin, and correcting the value for the internal standard recovery. All the stilbene standards were obtained from Extrasynthese (Genay Cedex, France). Data were analyzed by the Waters BreezeTM Chromatography Software (Version 3.30). The limits of detection (LOD) and quantification (LOQ) were calculated according to the procedure described by Shrivastava and Gupta (2011).

Statistical analysis

The results were evaluated by two-way analysis of variance (ANOVA), and the mean values were analyzed by Tukey's test using the software CoStat version 6.400 (CoHort Software 798, Monterey, CA, USA).

Results

Chemical characterization of chitosan: the commercial chitosan used in the present paper revealed a molecular weight (MW) of 173 (± 13) kDa and a degree of acetylation (DA)
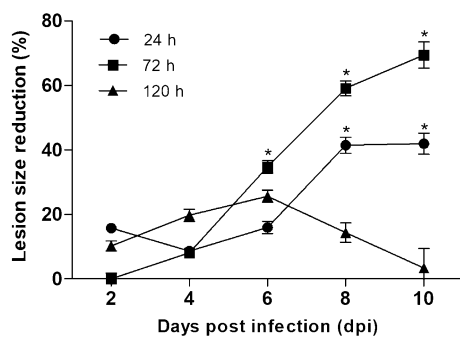
Fig. 1 Results of preventive application of chitosan. Lesion size reduction measured at different days post-infection (dpi) on leaves inoculated with *B. cinerea* 24, 72, and 120 h after chitosan treatment. Data were reported in percentage of disease reduction compared with the untreated leaves (Bc). Values represent the mean ± SD of triplicate assays. A statistical test comparing treated and untreated plants was performed for each observation time point. Asterisk indicates that values are significantly different ($P < 0.05$) according to Tukey's test

Fig. 2 Results of preventive application of chitosan. Average lesion diameter of *Botrytis cinerea* infection measured on untreated leaves (Bc) and on leaves treated with chitosan 24 h before *B. cinerea* inoculation (Chit + Bc) at different days post-infection (dpi) observed in the in vitro and in vivo assays. Values represent the mean ± SD of triplicate assays. A statistical test comparing treated and untreated plants was performed for each observation time point. Results with different letters are significantly different at 5% using Tukey's test

of 17.3 (± 2.5) % (Supplementary Fig. 1). Compared to the MW of chitosan used by Aziz et al. (2006) and Trotel-Aziz et al. (2006), which was below 10 kDa, the biopolymer used in the present study can be considered a high MW chitosan. This aspect is important as it increases the viscosity of the chitosan solution; higher viscosity allows the formation, on the leaf surface, of a chitosan layer, which can act as a physical barrier, helping to protect against fungal attack (Ait Barka et al. 2004).

In addition, the chitosan showed a relatively low acetylation degree, which is important for both the solubility of the polymer (the chitosan was totally soluble when dispersed at 2.5% in a solution of acetic acid 1%) and the fungicidal activity. As a matter of fact, the antifungal activity is proportional to the number of free amino groups, and the effect is explained by the interaction of positively charged chitosan molecules (cationic properties) with the negatively charged pathogen surface, leading to damage of the pathogen cell due to an increase in the cell membrane permeability (Rabea et al. 2003).

Direct antifungal properties of chitosan against Botrytis cinerea

Antifungal assays on nutrient agar medium revealed that the addition of chitosan solution affects the *B. cinerea* development (Supplementary Fig. 2). On the controls, the mycelium growth started at 1 dpi and reached the total coverage of the plates at 5 dpi. From 3 dpi, significant differences were observed between all the plates with the chitosan solutions and the controls. In detail, 2 and 3 g/L of chitosan allowed a reduction of 40% on the mycelium growth respect to controls, whereas with the solutions 0.5 and 1 g/L the average
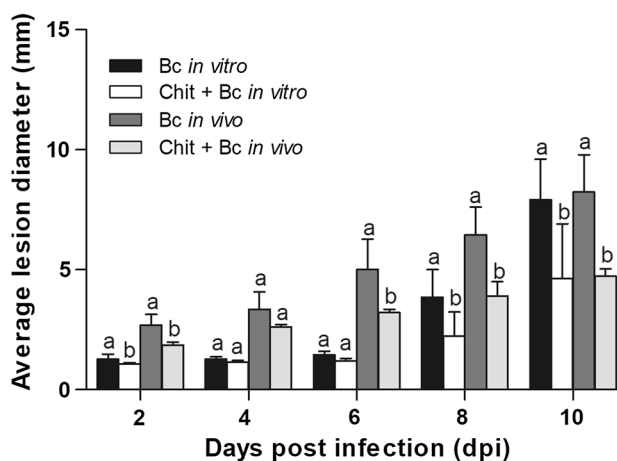
reduction was 22%. Increasing concentrations of chitosan showed clearly an inhibitory effect of the polymer on the mycelium growth. The IC_{50} after 120 h of incubation was 3 g/L.

Chitosan treatment reduced B. cinerea infection on grapevine

A preliminary test was performed to exclude any phytotoxic effect caused by chitosan treatment on grapevine leaves. The highest dose of chitosan application (1 g/L) did not show phytotoxic effects.

Different assays were performed inoculating *B. cinerea* on grapevine detached leaves 24, 72, and 120 h after chitosan treatment. The highest protection levels were reached for treatments carried out 24 and 72 h before *B. cinerea* inoculation. In both assays, the highest percentage of disease reduction, compared to controls, was observed at 10 dpi, with a reduction of necrotic lesion diameter of 41% and 69% for treatments performed 24 and 72 h before *B. cinerea* inoculation, respectively (Fig. 1).

The protection level induced on grapevine detached leaves (in vitro) by chitosan treatment, carried out 24 h before *B. cinerea* inoculation, was compared to that obtained on the whole plants (in vivo). The evolution of *B. cinerea* infection was slower on assays performed on detached leaves compared to assays with plants, albeit a similar protection effect induced by chitosan treatment was detected in both trials (Fig. 2). For both assays, treated leaves showed a significant reduction of the

average lesion diameter from two days after *B. cinerea* inoculation, in comparison with untreated leaves (df 1, F 8.01, *P* 0.01 and df 1, F 21.72, *P* 0.0003 for in vitro and in vivo assay, respectively). As for detached leaves, the highest protection induced by the treatment on the plants was reached at 10 dpi, with 43% reductions of lesion diameter compared to the positive control.

Treatment with chitosan modulated the expression of some grapevine defense-related genes

The expression level of a set of defense marker genes was evaluated to investigate whether the protection induced by chitosan application could derive from the elicitation of grapevine defense responses.

Activation of defense response in plants is mediated by an interconnected network of signal transduction pathways depending mainly by hormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (Et). Modulation of the expression level of six genes involved in hormone-mediated signaling was investigated at different time points after chitosan treatment of grapevine plants (Fig. 3a). Generally, few changes in gene expression were observed for all the investigated genes at one day after treatment. The more interesting results emerged from 3 to 6 dpt, when it was observed the downregulation of *NPR1* and *EDS1*, two marker genes of SA-mediated signaling, and the upregulation of *JAZ1.1*, *LOX9* and *LOX13*, three genes involved in the JA-mediated defense response, beside that of *ACO*, a key gene of Et-mediated response. Later, at 9 dpt differences in genes expression level between treated and untreated leaves became less evident. Moreover, transcription of *GST1*, a gene involved in the regulation of the redox status, was transiently upregulated one day after treatment, but it was strongly downregulated from 3 dpt. Contrariwise, the expression level of *WRKY1* was slightly enhanced until 9 dpt.

The expression pattern of five *PR* genes, which are reliable defense markers in grapevine, was investigated on plants treated with chitosan (Fig. 3b). The expression of most *PR* genes was downregulated after chitosan treatment, except for *PR5*, encoding a thaumatin-like protein, whose transcription was substantially higher at 1 dpt and remained high thereafter, up to decrease at 9 dpt.

One day after the treatment, *PAL* and *STS* expressions, two key genes of the phenylpropanoid pathway, were induced by chitosan (Fig. 3c). Successively, the transcription of both genes was slightly downregulated at 3 dpt, and then, from six days, there was an increase in the transcription of *PAL*, while the mRNA level of *STS1* continued to decrease until 9 dpt.
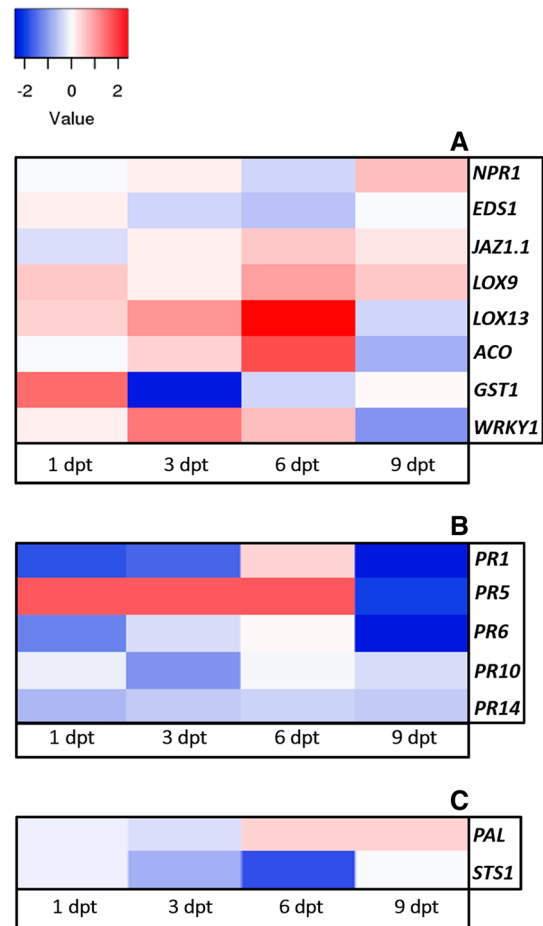

Fig. 3 a Transcript levels of genes involved in different signaling pathways on grapevine induced after chitosan treatment, **b** transcript levels of some PR genes on grapevine after chitosan treatment, **c** transcript levels of marker genes of the phenylpropanoid pathway on grapevine after chitosan treatment. Each column represents the time in days post-treatment, and each row represents one gene. A tree color scale was used to show fold induction of each gene (log transformed). The fold induction values were normalized to the reference genes *GAPDH* and *COX* and to untreated leaves as the control samples

Treatment with chitosan-induced transient accumulation of phytoalexins

The accumulation of some stilbene compounds following chitosan treatment was investigated on the same leaves collected for transcriptomic studies. Among the three searched stilbenes, it was possible to quantify *trans*-resveratrol, while both *trans*-piceatannol and *trans*-ε-viniferin were undetectable. Significant difference between treated and untreated plants was observed only at 3 dpt, with a higher accumulation of *trans*-resveratrol detected on treated plants (df 1, F 10.63, *P* 0.036) (Fig. 4).

Discussion

Chitosan is a highly investigated biopolymer with well-known antimicrobial properties. This antimicrobial effect is largely influenced by the molecular weight, the degree of acetylation (DA) as well as the derivatization and preparation methods used (Verlee et al. 2017). On grapevine, chitosans with a low MW (1.5 kDa) and with DA lower than 20% were identified as the best inducers of plant defenses (Aziz et al. 2006). The characterization of chitosan used in this work revealed that it had a MW of 173 kDa and a DA of 17% (chitosan 173/17). According to the classification reported by some authors, a chitosan with polymer dimension of 173 kDa should be classified as low MW chitosan when compared to native polymers with MW higher than 300 kDa (Verlee et al. 2017). However, in terms of solubility and viscosity, a very large difference can be observed between the LMW chitosan's (less than 10 kDa) used in the majority of papers reporting antifungal properties and that used in the present paper (173 kDa). For this reason, considering its physical properties, we classified this chitosan as HMW. The degree of acetylation (17%) was similar to that which showed the best antifungal activity in previous experiments (Aziz et al. 2006). Indeed, it has been suggested that the presence of at least a small percentage of acetylated groups (i.e., chitin portion) could help a better binding by the receptors on the plasma membrane.

Chitosan solution (173/17) showed a direct fungistatic effect against *B. cinerea* that confirmed data reported by other authors. Ait Barka et al. (2004) found a 44% mycelium reduction when incorporating in the PDA medium 10 g/L of chitogel (a commercial preparation of chitosan, but no information about chitosan molecular weight and concentration was reported). Reglinski et al. (2010) found an IC_{50} of 0.175 g/L adding a commercial preparation of chitosan (Armour Zen) in the liquid medium. Even in this case the molecular weight of chitosan was not reported. Only Aziz et al. (2006) excluded a direct effect of chitosan on *B. cinerea* observing only slight growth inhibition with chitosan concentration up to 0.3 g/L. However, the chitosan used in those experiments exhibited very low molecular weight (1.5 kDa) in comparison with the chitosan used in the present experiment (173 kDa).

Assays performed on grapevine leaves and plants revealed that chitosan solution (173/17) conferred a good level of protection against *B. cinerea* (Fig. 2). The protective effect of chitosan was reported by many authors on different plant species. Povero et al. (2011) demonstrated the ability of LMW chitosan in protecting Arabidopsis leaves against *B. cinerea.* On grapevine, chitosan, dissolved in acetic acid, reduced post-harvest gray mold of table grape (Romanazzi et al. 2009). Ait Barka et al. (2004) reported the efficacy against *B. cinerea* of foliar application of Chitogel on V*itis vinifera*. Aziz et al. (2006) showed a 50% reduction of *B. cinerea* lesion diameter after application of 0.05 g/L of low molecular weight chitosan. Reglinski et al. (2010) observed a significant effect on *B. cinerea* development only after application of 5 g/L of chitosan. All these authors started the infection 6–48 h after the chitosan application, because it is well known that chitosan is a resistance inducer and needs a delay in order to show its effect. It has been shown that chitosan, mainly at LMW, elicits on grapevine a variety of defense reactions, such as transient increasing of LOX, PAL and chitinase activities (Trotel-Aziz et al. 2006e). Recently, it has been revealed that chitosan hexamer induced on *V. vinifera* cell suspensions a rapid expression of some defense genes, including *STS* and *PAL* (Brulé et al. 2019).

In the present work, after treatment with chitosan many genes involved in hormone-signaling pathways resulted slightly modulated (Fig. 3a). Interestingly, the higher modulation observed six days after the treatment revealed an induction of the JA/ET-mediated response and a repression of the SA-mediated signaling. In particular, *LOX-13*, a gene encoding a lipoxygenase that catalyzes the initial step of jasmonate formation in plants, and *ACO*, a gene involved in the synthesis of a precursor of ET, were significantly upregulated. JA, SA, and ET are central players in mediating responses to pathogens and wounds. SA is usually associated with response to biotrophic pathogens, whereas JA/ET are most often thought to function in response to wounding and to necrotrophic pathogens (Glazebrook 2005). Several reports demonstrated that the application of chitosan to many plant species, including rice, led to a rapid increase in the JA content through the activation of the octadecanoic pathway (Doares et al. 1995; Rakwal et al. 2002; Povero et al. 2011). Enhanced expression of *LOX* genes was reported by many authors on grapevine in response to elicitors, such as laminarin, and this result agrees with the increased lipoxygenase activity reported on grapevine leaves after treatment
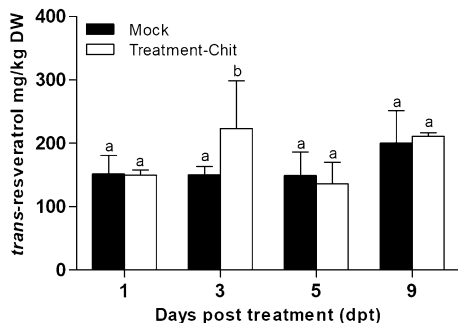

Fig. 4 Content of *trans*-resveratrol measured on grapevine leaves treated with chitosan compared with untreated ones at different days post-treatment (dpt). Results with different letters are significantly different at 5% using Tukey's test

with LMW chitosan (Aziz et al. 2003; Trotel-Aziz et al. 2006). The main significative event observed one day after chitosan treatment was a higher expression of *GST1*, followed by a marked downregulation of the same gene. *GST1* encodes for an enzyme which takes part in the detoxification of elicitor-generated oxidants, and the increase in its expression was reported in response to the oxidative burst (Levine et al.1994; Mauch and Dudler 1993; Vanacker et al. 2000).

Despite some transcriptomic modulation of several genes involved in signaling pathways induced by chitosan, the only significant event observed at downstream level was the upregulation of a *PR5* gene until six days after treatment (Fig. 3b). On the opposite, the transcription of the other *PR* genes was generally downregulated or otherwise it was at the same levels on treated and untreated leaves. Unexpectedly, the expression of two genes involved in the biosynthesis of stilbenic phytoalexins showed low modulation after chitosan treatment, despite the finding of a transient accumulation of *trans*-resveratrol in treated plants at 3 dpt (Fig. 4). Aziz and collaborators (2006) reported a peak of *trans*-resveratrol accumulation in grapevine leaves at 48 h after treatment with chitosan with dependency on DA and MW. The highest phytoalexin accumulation was triggered by chitosan with a DA from 2 to 20% and a MW from 1.5 to 3 kDa.

Data reported in the present work highlight that the addition of chitosan (173/17) activated the grapevine response with some delay (highest modulation of signaling genes at 6 dpt and highest accumulation of *trans*-resveratrol at 3 dpt). Therefore, it can be supposed that a partial degradation of chitosan polymers, caused by the hydrolytic activity of plant chitinases, is needed to induce a substantial grapevine defense response. However, it was observed a significant reduction of the spreading of necrotic lesions caused by *B. cinerea* already from one day after chitosan treatment. Therefore, it is likely that chitosan applied on grapevine leaves acts also as a direct inhibitor of *B. cinerea* development. Moreover, as previously suggested by Ait Barka et al. (2004), beside the induction of defense mechanisms, chitosan, thanks to its filmogenic property, may also act as a physical barrier to fungal attack. It has to be taken into consideration, however, that filmogenic properties of chitosan are strictly dependent on its molecular weight, and the low molecular weight chitosan generally used for foliar application loses this property.

Conclusions

Data presented in this paper show that the good level of protection for grapevine leaves against *B. cinerea*, conferred by chitosan (173/17), could be the result of three properties of chitosan. Indeed, chitosan can act on grapevine leaves as a physical barrier to fungal attack and directly by affecting

fungal growth, mainly during the first days after treatment, and, successively, as an inducer of grapevine defense reactions.

Compliance with ethical standards

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