Oligosaccharides from *Botrytis cinerea* and Elicitation of Grapevine Defense

32

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Contents

Abstract

An extract from *Botrytis cinerea* culture filtrate was sprayed on grapevine plants (*Vitis vinifera*) to investigate its potential to stimulate defense reactions. The extract triggered the induction of genes encoding pathogenesis-related (PR) proteins as chitinases (*CHIT*), polygalacturonase-inhibiting protein (*PGIP*), serine proteinase inhibitor (*PIN*), and enzymes involved in phytoalexin synthesis as phenylalanine ammonia-lyase (*PAL*) and stilbene synthase (*STS*). Correlated to the up-regulation of these latter genes, stilbene content increased in treated leaves. Consequently, treatment of grapevine leaves with the fungal extract triggered protection toward *Plasmopara viticola* and *Erysiphe necator*,

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the causal agents of grapevine downy and powdery mildews, respectively. Disease severity was significantly reduced in elicited plants, approximately 61 % for downy mildew and 83 % for powdery mildew. This approach could represent a valuable strategy to protect grapevine from diseases as an alternative or complementary method to the use of pesticides.

Keywords
Elicitor • *Vitis vinifera* L. • Gray mold • *Plasmopara viticola* • *Erysiphe necator* • Stilbenes • Protection

Abbreviations

Bc	*Botrytis cinerea*
BTH	Benzothiadiazole
CHIT	Chitinase
Ct	Cycle threshold
DP	Degree of polymerization
DW	Dry weight
HPLC	High-performance liquid chromatography
INA	2,6-Dichloroisonicotinic acid
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OGs	Oligogalacturonides
PAL	Phenylalanine ammonia-lyase
PG	Endopolygalacturonase
PGIP	Polygalacturonase-inhibiting protein
PIN	Serine proteinase inhibitor
PR	Pathogenesis-related
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SA	Salicylic acid
STS	Stilbene synthase
TFA	Trifluoroacetic acid

1 Introduction

Grapevine (*Vitis vinifera* L.) is susceptible to many diseases, especially fungal ones such as gray mold (*Botrytis cinerea*), downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*), and dieback (*Eutypa lata*). To defend themselves against pathogens, plants have evolved several mechanisms. Passive defenses as structural barriers and preformed antifungal compounds help to delay the infection process but are insufficient. So plants have also developed active defense mechanisms leading to the accumulation of antimicrobial compounds such as phytoalexins (stilbenes in *V. vinifera*) and PR proteins (Jeandet et al. 1995; Adrian et al. 1996; Dufour et al. 2013; Rivière et al. 2012).

These active mechanisms are induced only if the plant has recognized the attack by the perception of signal molecules also called elicitors. Elicitors can be abiotic or biotic (Sticher et al. 1997; Mauch-Mani and Métraux 1998; Pieterse et al. 1998; Zimmerli et al. 2000; Ton et al. 2005; Delaunois et al. 2014). "Abiotic elicitors" can be physical stimuli like wounding or UV light exposure (Gus-Mayer et al. 1998; Douillet-Breuil et al. 1999; Colas et al. 2012) or chemicals like aluminum chloride or phosphite (Saindrenan et al. 1988; Borie et al. 2004; Bock et al. 2012). "Biotic elicitor" usually refers to molecules secreted by microorganisms, derived from the cell walls of fungi, bacteria, and host plants (Côté and Hahn 1994; Ebel and Cosio 1994; Guo et al. 2011) or from seaweed (Bouarab et al. 1999; Klarzynski et al. 2000; Cluzet et al. 2004; Trouvelot et al. 2008; Jaulneau et al. 2011). Oligosaccharides were among the earliest elicitors that have been characterized (Ebel 1998), and many cell wall poly- or oligosaccharides, such as microbial β-glucans and chitin-derived oligomers or plant pectin-derived oligogalacturonides (Ebel 1998; Côté et al. 1998; Aziz et al. 2007), exhibit elicitor activities on defense responses across different plant species (Cardinale et al. 2000; Mithofer et al. 2000; Inui et al. 1997; De León and Montesano 2013). Moreover, β-glucans from fungal origin have been reported to enhance phytoalexin production in soybean (Sharp et al. 1984) and protection against viruses in tobacco (Rouhier et al. 1995; Fu et al. 2011). As the intensive use of phytochemicals to protect plants triggers the emergence of pesticide-resistant strains (Leroux et al. 1999; Gressel 2011; Corio-Costet 2012) and as a current attention was paid to the environment, alternative treatments have to be developed. Application of elicitors could be an attractive approach to control plant diseases (Lyon et al. 1992; Benhamou and Nicole 1999; Mishra et al. 2012; Dufour and Corio-Costet 2013).

In a search of new elicitor compounds of grapevine defenses, a preparation of *Botrytis cinerea* extract was performed. *B. cinerea* is one of the most prevalent fungi that damage plants, and in reaction, plants have developed an ability to recognize its elicitors and to react to them strongly by inducing defense responses, as in *Vitis* spp. (Langcake and Pryce 1976). Eliciting properties could be attributed to saccharidic and/or proteic compounds secreted by the fungus. An endopolygalacturonase (BcPG1), purified from culture filtrates of *B. cinerea* and known as a virulence factor participating in this fungus pathogenicity, exhibited elicitor activity on defense responses in grapevine (*V. vinifera* cv. Gamay) (Ten Have et al. 1998; Poinssot et al. 2003). The authors demonstrated that the protein itself, rather than its enzyme activity, was responsible for defense response activation. Another hypothesis of this elicitor activity could be the presence in the filtrate of extracellular polysaccharides such as glucans and rhamno-galacto-mannans (Fanizza et al. 1995). Oligosaccharide fragments have previously been shown to elicit resistance reactions in several plants (Guo et al. 2011; Aziz et al. 2007), like chitooligosaccharides in wheat and grapevine leaves (Vander et al. 1998; Aziz et al. 2006).

The aim of this study was to determine whether exogenous application of a crude preparation of *B. cinerea* (*Bc*) culture filtrate on susceptible grapevine plants (*V. vinifera* L. cv. Cabernet Sauvignon) was able to induce protection toward

major pathogens. After treatment of plants by *Bc* extract, the expression of defense-related genes encoding enzymes involved in the phenylpropanoid pathway (*PAL* and *STS*) and PR proteins (*CHIT1a*, *CHIT3*, *CHIT4c*, *PIN*, and *PGIP*) was monitored by real-time quantitative RT-PCR. Stilbene content in leaves was evaluated by high-performance liquid chromatography (HPLC) analysis. Protection experiments toward downy (*P. viticola*) and powdery (*E. necator*) mildew infections were undertaken on detached leaves of *Bc* extract-treated plants.

2 Sources, Preparation, Usage

2.1 *Botrytis cinerea* Extract Preparation

The ascomycete pathogen *B. cinerea* (strain 163, virulence factor 6.19) was friendly provided by Dr. M. Fermaud (INRA, Villenave d'Ornon, France). The fungus was grown on malt agar medium at 20 ± 2 °C with a 12 h photoperiod to obtain conidia. For elicitor preparation, the fungus was grown in liquid medium as previously described (Fanizza et al. 1995). Conidia from 10-day-old fungal cultures on solid medium were scrapped from the surface of cultures and suspended in water containing 0.05 % Tween 20®. Aliquots of the conidial suspension were added to 2 L Erlenmeyer flasks containing 800 mL of Czapek-Dox medium to give a final concentration of $2.5.10^4$ conidia.mL^{-1} medium. The fungus was grown at 20 ± 2 °C in still cultures in darkness for 4 weeks. Culture filtrate was collected after mycelium removal by filtration and autoclaved for 1 h at 120 °C. This autoclaved filtrate contained 8.5 g of β-glucan.L^{-1} including DP6 (50 %), DP5 (32 %), and DP2–3 (18 %).

Concentration of the extract was expressed in g of glucose equivalents.L^{-1} according to the phenol-sulfuric method (Dubois et al. 1951). Before plant treatment, the solution was diluted in sterile water and the wetting agent Triton X-100® was added at 0.1 % (v/v). The extract was sprayed on plants at a final concentration of 2 g of glucose equivalents.L^{-1}.

3 Biological Properties

3.1 Plant Material, Treatment, and Infection Procedures

Plants of cultivated grapevine (*V. vinifera* L. cv. Cabernet Sauvignon), kindly supplied by Dr. M. F. Corio-Costet (INRA, UMR Santé Végétale, Villenave d'Ornon, France), were propagated from woodcuttings in a greenhouse. Plants were grown under controlled conditions at 25/20 °C day/night temperature, with 75 % relative humidity and a 16 h photoperiod (350 µmol.m^{-2}.s^{-1}). Two-month-old plants with 10–12 leaves were used, and a solution containing either water or the *B. cinerea* extract preparation, both supplied with the wetting agent Triton X-100® at 0.1 % (v/v), was sprayed on all the leaves. In the case of further inoculation tests, a negative control (water) and a positive one (commercial product Aliette® (Bayer)

containing fosetyl-Al) were introduced in the experiment. Twelve plants were used per treatment and each experiment has been repeated three times. At several times after treatment, leaves were collected for analysis. Fungal inoculation tests were performed on 48 h treated detached leaves from the upper part of the shoots. *P. viticola* and *E. necator* fungal strains were kindly provided by the UMR Santé Végétale of the INRA, Villenave d'Ornon, France. *P. viticola* was maintained on grapevine leaf-disk and subcultured two times before the assay. Sporangia were collected and suspended in demineralized water. Thoroughly rinsed, cleaned and dried leaves were placed upside down on moist paper filter in Petri dishes. Lower surfaces of the leaves were inoculated with the fresh prepared sporangia suspension and incubated for seven more days at 22 °C under a 16 h photoperiod. For infection with *E. necator*, detached leaves were cleaned, decontaminated with NaOCl, rinsed, and dried. Leaf disks were deposited lower side down on sterile agar plates and placed at the bottom of a Plexiglas settling tower (Délye and Corio-Costet 1998). Conidia were blown in at the top from leaves displaying spores. Inoculated leaves were incubated for 14 days at 22 °C under a 16 h photoperiod.

3.2 Defense-Related Gene Expression in Grapevine Plants

Most elicitor- and/or pathogen-induced genes that have been characterized in grapevine correspond to genes encoding PR proteins (Dufour et al. 2013; Busam et al. 1997; Davies and Robinson 2000; Jacobs et al. 1999; Robert et al. 2001; 2002; Belhadj et al. 2006, 2008a, 2008b; Bavaresco et al. 2012; Lambert et al. 2013) or enzymes involved in the synthesis of stilbene phytoalexins (Dufour et al. 2013; Belhadj et al. 2006, 2008a, 2008b; Bavaresco et al. 2012; Lambert et al. 2013; Melchior and Kindl 1990, 1991; Sparvoli et al. 1994; Wiese et al. 1994).

In this study, grapevine foliar cuttings of a susceptible cultivar (Cabernet Sauvignon) were sprayed with a fungal preparation derived from *B. cinerea* at the previously determined optimal concentration of 2 g of glucose eq L^{-1}. During the 48 h following plant treatment, expression of several defense-related genes encoding enzymes implicated in the synthesis of stilbenes (*PAL*, *STS*) and genes encoding PR proteins (*CHIT1a*, *CHIT3*, *CHIT4c*, *PGIP*, *PIN*) was analyzed in leaves. This expression was monitored by RT-qPCR using specific primers (Table 1) with an actin gene as internal standard. The quantification of mRNA expression levels was carried out as follows: total RNA was extracted from frozen leaves as described by Chang et al. (1993). Contaminating DNA in the RNA preparation was removed by DNase I (Promega Corp.) and a phenol/chloroform/isoamylic alcohol mixture was done to remove the DNase. Total RNA was checked for its integrity by electrophoresis and 2 µg was reverse-transcribed with oligo (dT) (ImProm-II™ reverse transcription System, Promega Corp.). For the determination of the mRNA copy number of the genes of interest, real-time quantitative RT-PCR (RT-qPCR) was performed using the detection system MyiQ (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). PCR reactions were carried out in triplicates in 96-well plates by using SYBR Green I dye and the appropriate primers

Table 1 Gene accession numbers and corresponding primer sequences used for real-time quantitative polymerase chain reaction

Names	Accession numbers	Forward primer	Reverse primer
PAL	X75967	TGCTGACTGGTGAAAAGGTG	CGTTCCAAGCACTGAGACAA
STS	AF274281	GTGGGGCTCACCTTTCATT	CTGGGTGAGCAATCCAAAAT
CHIT1a	AJ291505	TTTTGTCCACTCTGCTATGGTG	CACAGAAAGATTTGGATGCTCA
CHIT3	AJ291507	ATAAGTTCATGGGCACTGCTCT	AGGTTAGTGGTGTTGCCAGAAG
CHIT4C	AY137377	GGCGACGAATCCATTTATGTT	CGGAACAAGGGTTTCATAATTC
PGIP	AF305093	ACGGAACTTGTTCCAGTTTGAT	CGATTGTAACTCACGTTCAGGA
PIN	AY156047	GCAGAAACCATTAAGAGGGAGA	TCTATCCGATGGTAGGGACACT
ACT	TC30205	TCAGCACTTTCCAGCAGATG	TAGGGCAGGGCTTTCTTTCT

couple. To check the specificity of the PCR reaction, melting curves were analyzed for each data point. Transcripts level was calculated as described by Arrieta-Montiel et al. (2001) with the use of a standard curve of known copy number for target sequence. The copy number of the sample was estimated by plotting the threshold cycle (Ct values) against the logarithm of the starting copy number. The absolute copy number for each sample was calculated from standard curves using the Ct value and normalized against grapevine actin gene as an internal control (Bézier et al. 2002) and control leaves as reference sample. The gene-specific primers are indicated in Table 1. Relative gene expression was obtained with the formula: fold induction $= 2^{-[\Delta\Delta Ct]}$, where ΔΔCt = [CtGI where ΔΔCt = [CtGI(unknown sample) – Ct VACT(unknown sample)] – [Ct GI (reference sample) – Ct VATC (reference sample)]. GI is the gene of interest and VACT is the grapevine actin gene used as internal control. The calibrator sample is the sample chosen to represent 1 × expression of the gene of interest (e.g., control leaves) (Winer et al. 1999).

In control leaves, no significant transcript accumulation of the different genes was detected during the 48 h incubation period.

Phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway, is involved in the biosynthesis of various defense-related compounds (phenolics, lignin, salicylic acid). In this pathway, downstream of PAL, stilbene synthase (STS) catalyzes the synthesis of resveratrol, the main phytoalexin produced by grapevine in response to biotic or abiotic stresses (Adrian et al. 1997; Coutos-Thévenot et al. 2001; Langcake and Pryce 1977a; Langcake and Pryce 1977b; Shen et al. 2012). Expression of *PAL* and *STS* was highly induced in response to *Bc* extract treatment. In elicited grapevine leaves, *PAL* and *STS* mRNA accumulation was transient and followed the same expression profile. Transcripts were detected at least 3 h after treatment, reaching a maximum around 12 h (300-fold increase for *PAL*, -600 for *STS*), and then rapidly returned to a basal level 24 h after treatment (Fig. 1a).

Bc extract treatment also led to the accumulation of mRNA transcripts of genes encoding PR proteins. The chitinase genes *CHIT1a*, *CHIT3*, and *CHIT4c* showed different expression patterns after treatment (Fig. 1b). Chitinases play a direct role in plant defense by degrading chitin, a major component of fungal cell walls, and thus inhibit hyphal growth (Collinge et al. 1993). An increased pathogen resistance was observed in transgenic plants overexpressing chitinases (Grison et al. 1996; Prasad et al. 2013; Chen et al. 2014). Moreover, chitinolytic breakdown products induce the production of phytoalexins and systemic acquired resistance (Brunner et al. 1998; Van Loon and Van Strien 1999). *CHIT1a* transcripts accumulated during the 48 h incubation with a sixfold maximum increase. *CHIT4c* mRNA transcripts accumulated the first. Induction of this gene started immediately after treatment, with two maxima (40-fold increase each) at 3 and 18 h, then decreased slowly until 48 h. *CHIT3* transcripts accumulation started later, with a peak around 18 h and a 60-fold increased level (Fig. 1b). *CHIT3* is inducible by pathogens such as *P. viticola* and *E. necator* or by chemicals such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), or benzothiadiazole (BTH) (Busam et al. 1997; Jacobs et al. 1999; Robert et al. 2001, 2002).

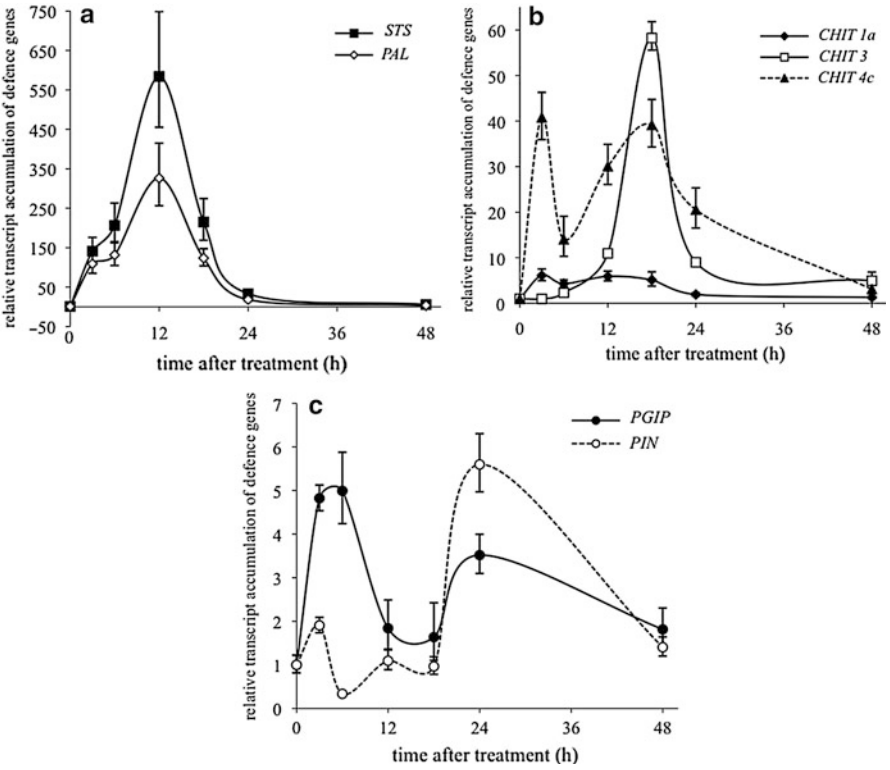

Fig. 1 Transcript accumulation of defense genes in grapevine leaves after treatment by *B. cinerea* extract. Expression profiles of genes encoding (**a**) a phenylalanine ammonia-lyase (*PAL*) and a stilbene synthase (*STS*), (**b**) chitinases (CHIT1a, CHIT3, CHIT4c), (**c**) a serine proteinase inhibitor (*PIN*), and a polygalacturonase-inhibiting protein (*PGIP*). Analyses were performed by RT-qPCR. Levels of transcripts were calculated using the standard curve method from triplicate data, with grapevine actin gene as internal control and no treated leaves (at time zero) as reference sample. Results represent the mean fold increase of mRNA level over no treated leaves, referred as the 1× expression level. In control leaves, the transcript level of defense genes was very low. Results represented are means of triplicate data ± SD of one representative experiment out of three

Bc extract treatment also induced a slight accumulation of polygalacturonase-inhibiting protein (*PGIP*) gene. PGIPs are plant defense proteins which reduce the hydrolytic activity of fungal endopolygalacturonases (PGs), preventing thus plant cell wall degradation and favoring the accumulation of oligogalacturonides (OGs) known to be elicitors of a variety of defense responses (Caprari et al. 1996; Rasul et al. 2012). In *Bc* extract-treated leaves, *PGIP* transcript accumulation increased from 3 h after the treatment with a first maximum (5-fold increase) as soon as 6 h followed by a second peak of lower intensity around 24 h (Fig. 1c).

Inhibitors of serine proteinases (*PIN*) have potent activity against plant and animal pathogens (Van Loon and Van Strien 1999; Revina et al. 2008). The RT-qPCR analysis revealed that the *PIN* gene was up-regulated in grapevine leaves

in response to fungal extract treatment. Accumulation began 5 h after treatment, peaked within 24 h (6-fold increase), and then decreased slowly (Fig. 1c).

3.3 Induction of Phytoalexin Biosynthesis

During the 2 days following *Bc* extract treatment, phytoalexin production in the upper leaves of the plant was monitored. In *V. vinifera*, the best characterized phytoalexins are stilbenes. These phenolic compounds are synthesized by stilbene synthase (STS), which catalyzes the synthesis of resveratrol (3,5,4′-trihydroxystilbene) (Wiese et al. 1994; Schöppner and Kindl 1984; Schröder et al. 1988; Richter et al. 2005). Resveratrol could be metabolized in piceid (glucosylation), viniferins (dimerization), or pterostilbene (methylation) (Bavaresco et al. 2012; Coutos-Thévenot et al. 2001; Pezet et al. 2004a). Identification and quantification of stilbenes were obtained by HPLC with fluorimetric detection, according to calibration curves of pure standards.

The *trans*-form content, which is the main form found in leaves, of five major stilbenic phytoalexins – resveratrol, piceid, ε-viniferin, δ-viniferin, and pterostilbene – was analyzed. Concerning standards, *trans*-resveratrol and *trans*-pterostilbene were purchased, whereas *trans*-piceid (*trans*-resveratrol 3-*O*-β glucoside) was purified from *V. vinifera* L. cell cultures as previously described (Waffo Teguo et al. 1996). *trans*-δ-Viniferin was synthesized by horseradish peroxidase from *trans*-resveratrol (Langcake and Pryce 1977b). *trans*-ε-Viniferin was purified from woody material and characterized by NMR and MS, as previously described (Pezet et al. 2003; Pawlus et al. 2012).

Except piceid, phytoalexins were undetectable in control leaves (Fig. 2a). *B. cinerea* extract treatment of grapevine leaves induced phytoalexin production with different time courses and levels of accumulation (Fig. 2).

Resveratrol was chronologically the first stilbene detected in leaves, 6 h after elicitor treatment. Accumulation of this compound during the 48 h analysis was quantitatively the most important among stilbenes produced. It accumulated transiently, peaked around 14 h after treatment (500 nmol g^{-1} dry weight (DW)), decreased until 24 h, and then remained stable to the end of the analysis (250 nmol g^{-1} DW) (Fig. 2a).

When resveratrol levels began to rise in elicited leaves, the other stilbenes were produced quite at the same time and peaked around 24 h. The level of piceid slowly increased to reach a maximum of approximately 200 nmol g^{-1} DW (fivefold level compared to control leaves) 24 h after treatment and then stabilized at a plateau. Both ε- and δ-viniferin syntheses were induced in elicited leaves. ε-Viniferin appeared earlier (around 6 h) and accumulated quantitatively to higher levels, a maximum around 350 nmol g^{-1} DW for ε-viniferin and 40 nmol g^{-1}DW for δ-viniferin. The levels of δ-viniferin rapidly decreased until 48 h (10 nmol g^{-1}DW), whereas ε-viniferin decreased slower (180 nmol g^{-1}DW at 48 h). Pterostilbene was also detected in treated leaves, but at relatively low levels (10 nmol g^{-1}DW). After reaching a maximum around

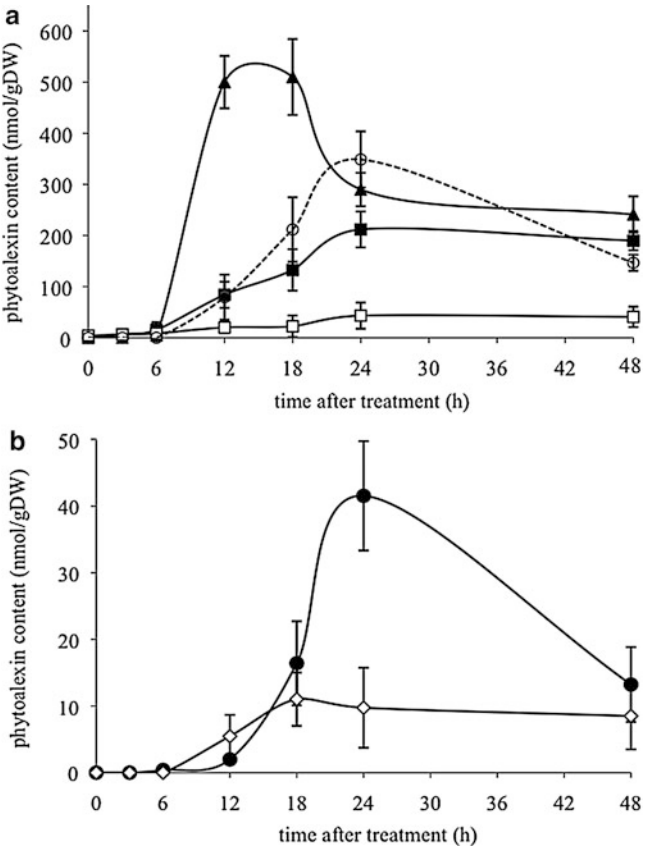

Fig. 2 Time course of several *trans*-stilbenes accumulation in *V. vinifera* (L.) cv. Cabernet Sauvignon leaves in response to *B. cinerea* extract treatment: (**a**) resveratrol (▲), piceid (■), and ε-viniferin (○). (**b**) δ-viniferin (●) and pterostilbene (◊). No stilbene except piceid was detected in untreated leaves (□). Values represent the mean ± SD of triplicate assays of one representative experiment out of three. Stilbenes from leaves were extracted by methanol, then pre-purified on a Sep-Pak® C18 cartridge to remove chlorophylls. Analysis of stilbenes was performed by HPLC on a C18 (5 µm) reverse-phase column (4 mm i.d. × 250 mm). Solvents used for the separation were (**a**) water with 2.5 % TFA and (**b**) 20 % A with 80 % acetonitrile. The elution program at 1 ml/min was as follows: 0–13 min, from 14 % B to 18 % B; 13–15 min, 18 % B; 15–34 min, from 18 % B to 32 % B; 34–36 min, 32 % B; 36–40 min, from 32 % B to 40 % B; 40–49 min, from 40 % B to 80 % B; 49–50 min, from 80 % B to 100 % B; 50–56 min, 100 % B. Fluorimetric detection was recorded at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 300$ nm

20 h, the level of pterostilbene remained constant until the end of the analysis (Fig. 2b).

Forty-eight hours after treatment, the amount of stilbenes in leaves has decreased for resveratrol and ε- and δ-viniferins, compared to the maximal levels they reached during the incubation period, whereas piceid and pterostilbene levels stabilized into a plateau from 24 h to the end of the analysis.

3.4 Protection Against *Plasmopara viticola* and *Erysiphe necator* by Bc Extract

To determine whether our extract induces disease protection in grapevine, *V. vinifera* cv. Cabernet Sauvignon leaves were treated with *Bc* extract.

An aqueous solution of the extract added with a wetting agent to improve penetration into the plant was sprayed on all parts of plants. Two days later, treated leaves were detached from plants and inoculated by *P. viticola* (downy mildew) and *E. necato*r (powdery mildew). As previous authors observed that older leaves from the bottom of the shoots are more resistant (Reuveni 1998), inoculation experiments were performed on young leaves from the upper part of the plant. Disease intensity was estimated 7 and 15 days post-inoculation for downy and powdery mildew, respectively, by measuring the infection rate.

As shown on Fig. 3, the validity of the test was checked according to the results obtained in Aliette® (fosetyl-Al)-treated leaves (3 g L^{-1}). Pretreatment with the fungal elicitor 2 days before inoculation induced a significantly strong reduction of the infection by both fungi, *P. viticola* and *E. necator*. The development of the pathogens was respectively reduced for about 61 and 83 % in leaves of plants pretreated by fungal elicitor extract compared to untreated plants. Aliette® protection rates were almost 100 % against *P. viticola* and about 86 % for *E. necator*.

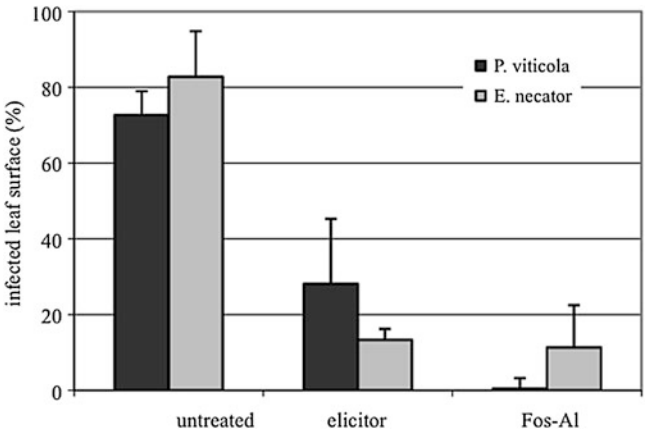

Fig. 3 Protection of grapevine detached leaves pretreated by *B. cinerea* extract against *P. viticola* (*black bars*) or *E. necator* (*gray bars*). Plants were sprayed with Bc extract or the known active Aliette® (fosetyl-Al) 2 days before inoculation, and disease assessment was done 7 days postinoculation for *P. viticola* and 14 days for *E. necator* and expressed as a percent infected leaf surface. Twelve plants were used per treatment. The experience was repeated twice with similar results. Values represent the mean ± SD of triplicate assays of one representative experiment out of two. Disease intensity was estimated by measuring the level of growth and intensity of fungal mycelium and sporulation on leaves. The contamination level was visually evaluated and expressed as percentage of total leaf area according to a 0–100 % scale with steps from 0 to 5 (the note zero corresponding to the absence of pathogen development). The intensity and aspect of spores formation was observed with microscope

4 Discussion and Conclusion

4.1 *Botrytis cinerea* Extract Induces Defense Responses in Grapevine

In *Bc*-treated plants, the expression of two of the three analyzed chitinase genes – *CHIT3* and *CHIT4c* – was rapidly detected after treatment and increased significantly, whereas *CHIT1a* expression remained relatively low during the 48 h incubation period. Indeed, grapevine chitinases are known to be inducible by various biotic stresses but differentially, according to the applied stress, the studied organ, and the grapevine cultivar (Busam et al. 1997; Jacobs et al. 1999; Robert et al. 2002; Aziz et al. 2003). *PGIP* and *PIN* mRNA transcripts accumulated in treated leaves. Levels of *PGIP* have been shown to correlate in several cases with an increased resistance of plants to fungi (De Lorenzo and Ferrari 2002; D'Ovidio et al. 2004; Wang et al. 2013). Increased expression of *PIN* gene was also observed. The prevailing role of serine proteinase inhibitors seems to be the control of endogenous proteinases during seed dormancy and protection against pathogens (Pautot et al. 1991). *PIN* mRNA and protein synthesis accumulation has been shown to occur in tomato leaves after treatment by plant-derived oligogalacturonides and fungal-derived chitosan oligosaccharides (Doares et al. 1995; Akagi et al. 2010).

PAL and *STS* genes were the two most rapidly and intensively up-regulated genes upon *Bc* extract treatment. Indeed, activation of phenylpropanoid metabolism is one of the most important resistance reactions in many plants (Dixon and Paiva 1995; Dixon 2011). In grapevine, previous studies showed that both *PAL* and *STS* genes were induced in leaves infected by *B. cinerea* (Belhadj et al. 2008a; Lambert et al. 2013; Bézier et al. 2002). Moreover, *PAL* and *STS* showed coordinated gene expression.

Consistent with the up-regulation of *PAL* and *STS* genes, we noticed an increased production of resveratrol and its derivatives – piceid, viniferins, and pterostilbene – in leaves of elicited plants. Resveratrol and its dimer ε-viniferin were quantitatively the major stilbenes produced in grapevine leaves in response to the fungal elicitor treatment. This result is in accordance with previous observations made in grapevine treated by biotic elicitors such as laminarin (Lambert et al. 2013; Aziz et al. 2003).

Resveratrol is the primary phytoalexin produced by a stilbene synthase after a stress (Jeandet et al. 2002, 2013). Once resveratrol synthesis began in leaves, consistently with the up-regulation of *STS* gene, its metabolization into the other compounds started. In our experiment, compared kinetics of accumulation of all compounds support the idea that resveratrol is the precursor of the phytoalexin grapevine phytoalexins. Furthermore, the highest resveratrol levels are reached around 12 h, whereas derivatives peaked later, at around 24 h.

The accumulation of ε- and δ-viniferins rapidly occurred in elicited leaves. Both compounds are highly fungitoxic and the presence of both viniferins in stressed

grapevine leaves has been correlated with enhanced protection against downy mildew (Pezet et al. 2004a, 2003, 2004b). The fungitoxic activity of δ-viniferin against zoospores of *P. viticola* is quite similar to the one of pterostilbene, the most toxic known stilbene. Our data showed that this latter compound was also detected in leaves of pretreated plants, even if the amounts seemed low compared to the other analyzed stilbenes. In the majority of grapevine cultivars, pterostilbene levels remain very low or undetectable and few studies reported its presence in response to elicitation. Resveratrol could be also glycosylated as piceid, which is protected from enzymatic oxidation (Regev-Shoshani et al. 2003). In our experiment, the accumulation of piceid was lower to the ones of resveratrol and ε viniferin. This could be explained by the fact that the plant does not need its presence because of its lower fungitoxicity. This stilbene could also represent a form of reserve or transport of resveratrol in the plant (Douillet-Breuil et al. 1999; Belhadj et al. 2008a). If the plant is stressed, the presence of basal piceid levels could constitute a pool of immediately usable resveratrol, which can rapidly be mobilized as a primary defense response.

4.2 *B. cinerea* Extract Treatment Led to an Increased Protection of Grapevine Against Downy and Powdery Mildews

Correlated to the induction of defense-related genes, *Bc* extract treatment of grapevine plants triggered enhanced protection toward two of the most deleterious grapevine fungal diseases. Indeed, pretreatment of plants reduced the development of *P. viticola* and *E. necator* by approximately 61 % and 83 %, respectively, compared to control leaves. In the case of *E. necator*, the protection obtained with *Bc* extract treatment was comparable to the one induced by Aliette®. We observed less difference in the efficiency against both pathogens in Aliette-treated plants than in *Bc* extract-treated ones. This probably could be explained by the fact that fosetyl-Al exhibits at the same time a direct antifungal activity (Dercks and Creasy 1989) and an indirect potentiation of phytoalexin biosynthesis (Adrian et al. 1996).

Bc extract triggers induction of PR genes. Activities of chitinases have been correlated in many grapevine cultivars with their observed field resistance to powdery mildew (Busam et al. 1997; Renault et al. 1996; Giannakis et al. 1998; Nirala et al. 2010), and constitutive accumulation of PR proteins in grape berries after veraison confers increased resistance to fungi such as downy and powdery mildews (Jacobs et al. 1999; Giannakis et al. 1998; Robinson et al. 1997; Tattersall et al. 1997; Salzman et al. 1998; Derckel et al. 1998; Kambiranda et al. 2014).

The protection acquired by grapevine leaves treated by the extract could be due to the up-regulation of *PAL* and *STS* gene expression and the consequent accumulation of the two quantitatively most produced stilbenes, resveratrol and its dimer ε-viniferin. Indeed, a higher tolerance to both powdery and downy mildews was observed in grapevine producing high levels of resveratrol (Dai et al. 1995; Malacarne et al. 2009). ε- and δ-viniferins have been shown to be the major

stilbenes produced in grapevine leaves infected by *P. viticola* (Pezet et al. 2003; Kortekamp and Zyprian 2003) and their synthesis has previously been shown to correlate to grapevine resistance to *B. cinerea* or *P. viticola* (Douillet-Breuil et al. 1999; Pezet et al. 2004a). Moreover, some studies suggest that both *P. viticola* and *E. necator* cannot detoxify resveratrol or suppress phytoalexin production in *Vitis* spp. (Romero-Pérez et al. 2001). In a recent work, Dufour et al. (2013) showed that the treatment of grapevine leaves with benzothiadiazole (BTH) led to a significant reduction in the development of these latter two fungi correlated with a significant increase only in pterostilbene contents (Dufour et al. 2013).

The enhanced tolerance of the treated grapevine leaves could thus be due to both the accumulation of PR proteins and phytoalexins (Belhadj et al. 2006, 2008a; Corio-Costet et al. 2012).

We noticed that the major stilbenes biologically active against pathogens were not present at sufficient levels in our experiments at the moment of the inoculation to counteract pathogen penetration. However, two of the most grapevine-destructive fungi, *P. viticola* and *E. necator*, exhibited a reduced development in treated plants. We can hypothesize that pretreatment of plants with the *Bc* extract certainly triggered a faster and stronger activation of defense responses to subsequent fungal attack as a potentiation.

We have shown the efficiency of an extract derived from *B. cinerea* culture filtrate. This fungal extract certainly contains elicitor compounds: *Bc* extract treatment showed a direct stimulating effect on the expression of defense-related genes which was correlated with the enhanced production of antimicrobial compounds. Consequently, induced plants exhibited a more efficient response to pathogen attack, with highly reduced infection rates. Experiments are in progress with purified fractions of *B. cinerea* filtrate in order to determine which molecules are responsible for the eliciting properties. Exploiting this strategy to control diseases and pests clearly meets with the current need toward sustainable agriculture at a lower environmental cost.

A few oligosaccharide products have been approved in many European countries, in the USA, and in several other countries for their application in agriculture. To our knowledge Iodus^{R} (a laminarin-based product acting as elicitor) approved as plant protection agent against several diseases and Elexa^{R} (a chitosan-based product) available as biological elicitors are the two main used products.

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