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Full Length Research Paper

Foliar application of systemic acquired resistance (SAR) inducers for controlling grape anthracnose caused by *Sphaceloma ampelinum* de Bary in Thailand

Inchaya Prakongkha[1], Mathukorn Sompong[1], Sopone Wongkaew[1], Dusit Athinuwat[3] and Natthiya Buensanteai[1,2]*

[1]School of Crop Production Technology, Institute of Agriculture Technology, Suranaree University of Technology, Nakhon Ratchasima, 30000 Thailand.
[2]Unité Mixte de Recherche Eco and Sols (Ecologie fonctionnelle and biogéochimie des Sols and des Agroécosystèmes), UMR 1222 INRA-IRD-SupAgro Montpellier, Place Pierre Viala, 34060 Montpellier cedex 01, France.
[3]Major of Organic Farming Management, Faculty of Science and Technology, Thammasat University, Pathumthani, 12121, Thailand.

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Chitosan and benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) are active-elicitors that induce protection in grapevine against several diseases. In this study, treatment of grapevine with chitosan and BTH provided protection to anthracnose, caused by *Sphaceloma ampelinum*. Chitosan and BTH treatment also increased chitinase, ß-1,3-glucanase and peroxidase activities levels in leaves over non-treated plants. Differential accumulation of these traits was more rapid and pronounced when chitosan and BTH treated plants were infected with *S. ampelinum*; this pattern indicating priming. The induced resistance by chitosan and BTH was also associated with increased production of salicylic acid (SA) in grapevine leaves, suggesting that SA-dependent signaling pathways are systemically triggered by these compounds. Apart from proteins with defense-related function, most of the proteins induced by chitosan and BTH were involved in defense mechanism, reflecting the strong direct positive effect that chitosan and BTH has on grapevine tolerance to anthracnose disease infection.

Key words: Anthracnose, grapevine, induce resistance, systemic acquired resistance (SAR) biochemical markers, *Sphaceloma ampelinum.*

INTRODUCTION

Grape anthracnose caused by *Spaceloma ampelinum* (telemorph: *Elisino ampelina*) is widely known in Thailand as scab (Pienpuck et al., 1993; Sompong et al., 2012). It is considered one of the most serious diseases of grapes in Thailand because of the favorable warm and wet climate. The fungus causes characteristic necrotic spots on leaves, petioles, tendrils, young shoots and berries. Severe infection can cause a range of symptoms from shot holes, shoot die-back, deformed leaves, blemished fruits, premature fruit drop, and vine decline (Poolsawat et al., 2008; Poolsawat et al., 2010). The disease is particularly severe during the rainy season starting from early May to late October when young berries start to develop. The disease is wide spread in all growing regions on most grape cultivars but table grapes are the most susceptible ones (Pienpuck et al., 1993; Poolsawat et al., 2008; Sompong et al., 2012). Chemical control by protective fungicides such as, dithiocarbamates alternated with systemic one, such as, benomyl or triazoles

*Corresponding author. E-mail: natthiya@sut.ac.th. Tel: 66 4 422 4204. Fax: 66 4 422 4351.

has been recommended but their application is often ineffective due to continuous rainfall during the rainy season. The sprayed chemicals are mostly washed off soon after the application and spray timing is difficult to be ascertained because of unexpected rainfall. Apart from such limitations, the target fungus can build up resistance to chemicals regularly used and the chemicals always leave their residues in the berries and environment making the application less desirable. Retractable plastic roofing over the vines has been proved effective in reducing the disease severity and making chemical control more efficient (Wongkaew and Boonkerd, 2010), but it is considerably costly and may be cost-effective only on the high-price table grapes.

In recent years, resistance inducers have been extensively evaluated as a means to control plant diseases based on the systemic acquired resistance (SAR) concept (Sticher et al., 1997; Vallad and Goodman, 2004). SAR is part of the plant innate defense system that could confer long-lasting protection against a broad range of pathogens (Jetiyanon et al., 1997; Kuć and Richmond, 1997; Eikemo et al., 2003; Durrant and Dong, 2004; Iriti et al., 2004; Godard et al., 2009; Verhagen et al., 2010; Verhagen et al., 2011; Graham and Myers, 2011; Iriti et al., 2011; Perazzoli et al., 2011; Hatem et al., 2012). Once resistance is induced, the plant acquires enhanced defensive capacity against subsequent infection by pathogens. SAR requires salicylic acid (SA) as a signal molecule and is associated with production and accumulation of pathogenesis-related (PR) proteins (Malamy et al., 1990; Vidal et al., 1997; Buensanteai et al., 2009; Mandal et al., 2009). The inducers could be both synthetic and natural compounds. Among the synthetic inducers, benzo (1,2,3)-thiadiazole-7-carbothionic acid S-methyl ester or acibenzolar-S-methyl (ASM), a functional homolog of SA has been extensively evaluated against grapevine fungal diseases (Perazzoli et al., 2008; Slaughter et al., 2008; Eschen-Lippold et al., 2010). Its application has been registered on many crops and in many countries (Friedrich et al., 1996; Godard et al., 1999; Vallad and Goodman, 2004; Eschen-Lippld et al, 2010; Graham and Myers, 2011). For natural compounds, chitosan biopolymers produced by deacetylation of chitin from crustracean exoskeleton, have been tested to be most effective in stimulating plant growth and building up resistance to many diseases (Sathiyabama and Balabramaian, 1995; Awadalla and Mahmoud, 2005; Manjunatha et al., 2009; El Hadrami et al, 2010). Mechanisms of chitosan-induced resistance have been recently revised and include rising of cytosolic H^+ and $Ca^{2+,}$ activation of MAP-kinases, callose deposition, oxidative burst, hypersensitive response (HR), synthesis of abscisic acid (ABA), jasmonate, phytoalexins and pathogenesis related (PR) proteins (Iriti and Faoro, 2009).

Even though there have been proves of effectiveness in using resistance inducers for controlling crop diseases, none of them have been tried on grape anthracnose. Chitosan has been widely used in Thailand but mostly as a plant growth stimulator and its potential as a disease resistance inducer has never been evaluate under the Thailand growing condition (Sompong et al., 2012). The aim of this research was to evaluate the effectiveness of these two different resistance inducers in controlling the grape anthracnose disease and to investigate their mode of action by monitoring the production of SAR relating chemicals after their application.

MATERIALS AND METHODS

Experimental condition and treatments

The experiment was conducted in a green house at Suranaree University of Technology, Nakorn Ratchasima, Thailand during August to October, 2011. The average temperature in the greenhouse was 30 ± 2 and 26 ± 2°C at day time and night time, respectively. Two month old cuttings of Black Queen grape, the most susceptible cultivar to anthracnose (Sompong et al., 2012) was used as experimental plants, and the single-spore-isolated GB4 , the most severe isolate of *S. ampelinum* (data not shown) was used as the pathogen. The experiment was arranged in a randomized complete block design with nine treatments replicated four times. The treatments consisted of foliar sprays of chitosan (Fluka chitosan from crab shell; low molecular weight) in 4% HCl and the dilute in deionized water at 1 000, 2 500 and 5 000 mgl^{-1}, ASM (Bion 50WG; 50% a.i.; Syngenta Crop Protection) at 50, 100, and 200 mg L^{-1}. All these inducers were sprayed onto the middle leaf of each plant until run off. The untreated control plants received only a deionized water spray. The treatments were similarly given to four sets of plants to correspond with the three sampling times of SAR chemical analyses that is, immediately after spraying that is, seven days and 15 days after spraying. The 4th set of plants was kept for disease scoring. At 10 days after the induction, the treated and control plants were inoculated with a spore suspension of *S. ampelinum* GB4 (4×10^6 spores ml^{-1}). The inoculation was done by spraying the spore suspension on to all leaves of each plant until run off.

SAR chemical analyses

At 0, 7 and 15 days after inducer application, the treated, the lower, and the upper leaves were collected from each treated and water-treated plants for SAR relating chemical analyses. After being detached, each leaf was cut into two halves and the three halved leaves were pooled and treated as 1 sample. Subsequently, they were cut to smaller pieces of about 1 square cm and thoroughly mixed before analysis samples were taken. Leaf tissues of the first half was analyzed for SA, while the second half was assayed for chitinase, ß-1,3-glucanase, and polyphenol oxidase (PPO) activities.

SA analysis

The method as described by Raskin et al. (1989) was used for the analysis. Pooled leaf tissue (0.5 g) from each replication were randomly sampled, frozen with liquid nitrogen and macerated in a cold mortar with 1 ml of extraction solution (90:9:1 volume of absolute methanol, glacial acetic acid, and distillate water). The extract was subsequently centrifuged at 12 000 g and 4°C for 15 min and the supernatant was collected for the analysis. To determine the SA content, 500 µl of the supernatant was mixed with

an equal volume of 0.02 M ferric ammonium sulfate, incubated at 30°C for 5 min and the absorbance at 530 nm was read by a spectrophotometer. The read absorbance was subsequently compared to those of the reference standard to obtain the actual amount of SA in the sample.

PR proteins and defense enzyme analyses

Total proteins from the second set of half leaves were extracted by the method as described by Buensanteai et al. (2009). The leaves were similarly cut and pooled as for SA analysis. Subsequently, 0.5 g of the frozen tissues from each replication were macerated in a cold mortar with 1 ml of extraction buffer (0.1M Tris-HCl pH7.0, 0.1M KCl, 1 mM phenylmethylsulfonyl fluoride, 10 ml/L Triton X100, and 3% polyvinylpyrrolidone). After being thoroughly vortexed, the extract was centrifuged at 12,000 *g* and 4°C for 30 min and the supernatant was collected for protein analysis using the standard Bradford protein assay (Bradford, 1976). After the assay, the protein homogenate from each replication was divided into three equal portions for enzyme activity determination.

Determination of chitinase activity

400 µl of the protein homogenate was mixed with an equal volume of 0.1% (w/v) colloidal chitin in 0.05M sodium acetate buffer pH 5.0 and incubated for 2 h at 37°C. The N-acetyl glucosamine (GlcNAc) produced from the reaction was determined by spectrophotometric reading at 585 nm.

Determination of ß-1,3-glucanase activity

62.5 µl of the protein homogenate was mixed with an equal volume of 4% (w/v) laminarin in 0.05M sodium acetate buffer pH 5.0 and incubated at 40°C for 10 min. (Pan et al., 1991). The reaction was stopped by adding in 375 µl of dinitrosalicylic acid and dipping in boiling water for 5 min. Glucose produced from the reaction was determined by reading the absorbance at 500 nm followed the method described by Pan et al. (1991) and Buensanteai et al. (2009).

Determination of PPO activity

Four dilutions of the homogenate of each replication were assayed for PPO activity using the procedure described by Thipayapong (1995). The dilutions were made by mixing the homogenate with the protein extraction buffer at the ratios 10:30, 20:20, 30:10, and 40:0 µl. Subsequently, 5 µl of catalase (84 unit μl^{-1}) was added to each dilution and incubated for 15 min before the absorbance at 412 nm was measured using a spectrophotometer. After the measurement, 1 ml of substrate solution (96 µM 2-nitro-5-thio-benzoic acid and 1.77 mM 4-methylcathechol in 0.1M Tris HCl pH 7.0) was added into the mixture of each dilution and the quinone produced from the reaction was monitored by measuring the absorbance at 412 nm every 5 s for 30 min. Conversion of the decreased absorbance into the PPO activity was done by linear regression as described by Thipayapong (1995).

Disease severity scoring

After fungal inoculation, plants in the four sets were observed for symptom expression and the first day when symptoms appeared in each treatment was recorded. Disease severity in each treatment was compared at five days after inoculation when plants in all treatments had shown the symptoms, which was at 15 days after the inducer application. Disease scoring 1 to 5 of Poolsawat et al. (2010) was used for the assessment where 1 = no symptom, 2 = less than 25 % of affected leaf area, 3 = 26-50 % of affected leaf area, 4 = 51-75 % of affected leaf area, and 5 = more than 75 % of affected leaf area.

Statistical analysis

Data for SA content and PR proteins and defense enzyme of the induced plants collected at three different dates, and disease scores assessed at 15 days after the induction were subjected to a one-way analysis of variance (ANOVA) separately using version 14 of SPSS program. The New Duncan's Multiple Range Test (DMRT) was used to separate the treatment means at $P \leq 0.05$.

RESULTS

Grapevine anthracnose disease severity assessment

In this experiment using grapevine cultivar Black Queens, foliar treatment with chitosan and BTH reduced the severity of anthracnose in the grapevine foliage, confirming that induction of systemic resistance had occurred. The results indicate that treatment with chitosan and BTH reduced the severity of anthracnose in leave by more 75 and 60%, respectively when compared to the sterile distilled water as negative control (Table 1, Figures 1 and 2).

SAR biochemical analyses

In this study, chitosan and BTH were evaluated for their ability to induce defense responses and related chemicals to protect grapevine from *S. ampelinum* infection. Grapevine treatment with chitosan and BTH triggered increased accumulation of biochemical markers associated with induced resistance mainly after *S. ampelinum* inoculation.

Salicylic acid analysis

Our results indicate that in grapevine plants of cultivar Black Queens treated with chitosan and BTH, salicylic acid level increased significantly seven days after treatment and much more seven days after challenge inoculation, reaching the maximum concentration of 13.08, and 12.15 µg g^{-1} fresh weights for chitosan and BTH (Table 2). By contrast, SA accumulation in non-treated, but pathogen-inoculated grapevine, was considerably lower (2.90 µg g^{-1} fresh weight).

PR proteins and defense enzyme analysis determination of chitinase activity

Our results indicate that in grapevine plants of cultivar Black Queens treated with chitosan and BTH, chitinase

Table 1. Effect of foliar treatment with chitosan and BTH on severity of anthracnose disease in grapevine cultivar Black Queens.

Treatment	Disease severity[1/]	
	Disease score[2/]	Disease symptom (Days after inoculation)
CHN 1,000 ppm	2.33ab[3/]	4
CHN 2,500 ppm	2.33ab	4
CHN5,000 ppm	1.33a	5
BTH 50 ppm	2.33ab	5
BTH 100 ppm	2.33ab	4
BTH 200 ppm	3.00b	5
Control	4.89c	2
F-test	**	
CV(%)	17.89	

[1/]Grapevine leaves were challenged with *S. ampelinum* strain GB4 or sterile distilled water 7 days after foliar treatment with chitosan and BTH; [2/]The disease score were including: 1 score = leaf area necrosis between 0-6%; 2 score = leaf area necrosis less than 25%; 3 score = leaf area necrosis between 26-50%; 4 score = leaf area necrosis between 51-75%; 5 score = leaf area necrosis over 75% of all leaf area; [3/]disease severity was evaluated seven days after challenging with *S. ampelinum* strain GB4 or sterile distilled water. Each value represents a mean of three replicate plants with two leave per plant. Mean in the column followed by the same letter are not significantly different according to the LSD test (α= 0.05).

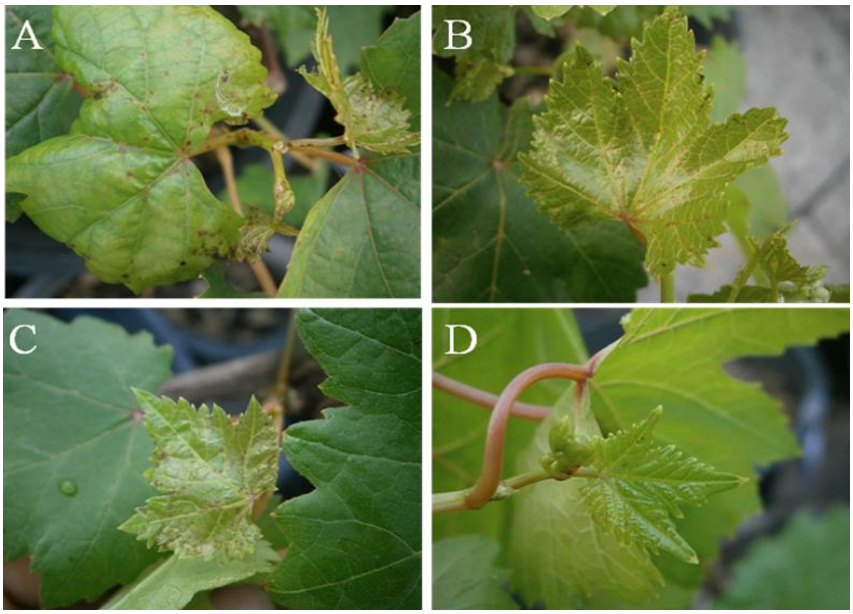

Figure 1. Anthracnose disease symptoms in leaves of grapevine cultivars Black Queens treated with water (A) or with chitosan at 1,000 ppm (B) 2,500 ppm (C) and 5,000 ppm (D).

activity level increased significantly 7 days after treatment and much more seven days after challenge inoculation with *S. ampelinum*, reaching the maximum concentration of 10.765 and 0.755 unit mg^{-1} protein respectively for chitosan and BTH (Table 3). By contrast, chitinase activity in non-treated, but pathogen-inoculated grapevine, was considerably lower (0.303 unit mg^{-1} protein).

Determination of ß-1,3-glucanase activity

Our results indicate that in grapevine plants of cultivar Black Queens treated with chitosan and BTH, ß-1,3 -glucanase activity increased significantly 7 days after treatment and much more 7 days after challenge inoculation, reaching the maximum concentration of 0.843, and 0.790 unit mg^{-1} protein respectively for

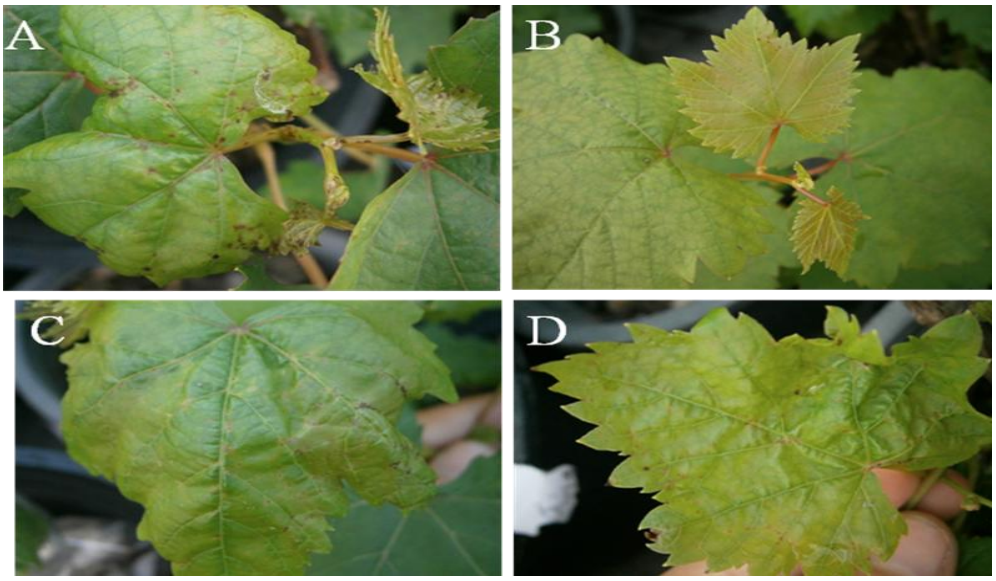

Figure 2. Anthracnose disease symptoms in leaves of grapevine cultivars Black Queens treated with water (A) or with BTH at 50 ppm (B) 100 ppm (C) and 200 ppm (D).

Table 2. Accumulation of salicylic acid in leaves of grapevine cultivars Black Queens with or without chitosan and BTH foliar treatments and after challenge inoculation with *Sphaceloma ampelinum.*

Treatment	Salicylic acid (µg g^{-1} fresh weight)[1/]		
	0	7 days	14 days
CHN 1,000 ppm	0.78[b2/]	3.57[e]	12.19[b]
CHN 2,500 ppm	0.60[a]	3.99[f]	10.58[b]
CHN 5,000 ppm	0.99[c]	2.98[d]	13.08[b]
BTH 50 ppm	0.79[b]	3.06[ef]	10.85[b]
BTH 100 ppm	1.04[c]	3.83[ef]	12.15[b]
BTH 200 ppm	1.46[e]	2.16[c]	11.54[b]
Control	1.21[d]	0.19[a]	2.90[a]
F-test	**	**	**
CV(%)	8.63	12.87	18.17

[1/]Salicylic acid accumulation was evaluated at 0 and 7 after treatment with chitosan and BTH or sterile distilled water (control) and at 7 days after challenging with *S. ampelinum* strain GB4 (14 days after treatment);[2/] each value represents a mean of three replicate plants with two leaves per plant. Mean in the column followed by the same letter are not significantly different according to the LSD test (α= 0.05)

chitosan and BTH (Table 4). By contrast, ß-1,3-glucanase activity in non-treated, but pathogen-inoculated grapevine, was considerably lower (0.467 unit mg^{-1} protein).

Determination of PPO activity

Our results indicate that in grapevine plants of cultivar Black Queens treated with chitosan and BTH, PPO activity level increased significantly seven days after treatment and much more seven days after challenge inoculation, reaching the maximum concentration of 11.227 and 10.153 µmol quinone formed min^{-1} mg^{-1} protein for chitosan and BTH, respectively (Table 5).

In contrast, PPO activity in pathogen-inoculated plants having no chitosan and BTH treatment was considerably lower. Non- chitosan and BTH treated plants of cultivar Black Queens exhibited a similar temporal change in PPO activity as chitosan and BTH treated plants. In this cultivar, foliar treatment with chitosan and BTH without pathogen inoculation caused only a slight elevation in PPO activity levels compared to the respective sterile distilled water control.

Table 3. Activity of chitinase in leaves of grapevine cultivars Black Queens with or without chitosan and BTH foliar treatment and after challenge inoculation with *S. ampelinum.*

Treatment	Chitinase activity (unit mg^{-1} protein)[1/]		
	0	7 days[2/]	14 days
CHN 1,000 ppm	0.133[3/]	0.396c	0.691c
CHN 2,500 ppm	0.135	0.417cd	0.726cd
CHN 5,000 ppm	0.132	0.444cd	0.755cd
BTH 50 ppm	0.130	0.423cd	0.710cd
BTH 100 ppm	0.131	0.434cd	0.732cd
BTH 200 ppm	0.133	0.451d	0.765d
Control	0.135	0.242a	0.303a
F-test	ns	**	**
CV(%)	15.44	9.75	5.10

[1/]Chitinase activity was evaluated at 0 and seven days after treatment with chitosan and BTH or sterile distilled water (control) and at seven days after challenging with *S. ampelinum* strain GB4 (14 days after treatment); [2/] Each value represents a mean of three replicate plants with two leave per plant. Mean in the column followed by the same letter are not significantly different according to the LSD test (α= 0.05).

Table 4. Activity of ß-1,3-glucanase in leaves of grapevine cultivars Black Queens with or without chitosan and BTH foliar treatment and after challenge inoculation with *S. ampelinum.*

Treatment	β-1,3-glucanase activity (unit mg^{-1} protein)[1/]		
	0	7 days	14 days
CHN 1,000 ppm	0.211[b2/]	0.470[d]	0.690[c]
CHN 2,500 ppm	0.270[cd]	0.493[de]	0.707[c]
CHN 5,000 ppm	0.223[b]	0.523[de]	0.750[cd]
BTH 50 ppm	0.312[g]	0.497[de]	0.747[cd]
BTH 100 ppm	0.282[de]	0.473[d]	0.790[cd]
BTH 200 ppm	0.213[b]	0.501d[e]	0.843[cd]
Control	0.195[a]	0.303[a]	0.467[a]
F-test	**	**	**
CV(%)	19.14	11.02	4.65

[1/] ß-1,3-glucanase activity was evaluated at 0 and 7 days after treatment with chitosan and BTH or sterile distilled water (control) and at 7 days after challenging with *Sphaceloma ampelinum* strain GB4 (14 days after treatment); [2/] Each value represents a mean of three replicate plants with two leave per plant. Mean in the column followed by the same letter are not significantly different according to the LSD test (α= 0.05).

DISCUSSION

In the present work, we show that the application of chitosan and BTH through foliar spray can induce resistance in grapevine against anthracnose caused by *S. ampelinum.* The induction of these resistances was dependent on the concentration of each elicitor, as already observed in a similar comparison carried out in a different pathosystem (Faoro et al., 2008). The most efficient induction was obtained when concentrations of 5 000 ppm for chitosan and 200 ppm for BTH were used. Our study, besides demonstrating for the first time that it is possible to induce resistance against *S. ampelinum* shows that this resistance is associated with salicylic acid and PR proteins synthesis, which are common plant biochemical responses associated to SAR activation (Sathiyabama et al., 1995; Mandal et al., 2009; Buensanteai et al., 2009; Cortes-Barco et al., 2010; Cohen et al., 2011). Interestingly, we found a major increase in SA accumulation in chitosan and BTH treated grapevine compared to the negative control after inoculation with *S. ampelinum.* The differential expression of the salicylic acid marker after pathogen challenge was similar to the induction of SA and jasmonic acid accumulation in grapevine by β-aminobutyric acid (BABA), that was potentiated by exposure to a callose formation preparation against *Plasmopara viticola*, the causal agent of downy mildew (Hamiduzzaman et al., 2005) and it is indicative of plant priming for resistance as defined by different authors (Sticher et al.(1997); Conrath et al. (2006); Trouvelot et al. (2007); Buensanteai et al. (2009) Archana et al. (2011)).

Table 5. Activity of PPO in leaves of grapevine cultivars Black Queens with or without chitosan and BTH foliar treatment and after challenge inoculation with *Sphaceloma ampelinum.*

Treatment	PPO activity (µmol quinone formed min^{-1} mg^{-1}protein)[1/]		
	0	7 days	14 days
CHN 1,000 ppm	3.061[ef2/]	2.987[ef]	8.297[e]
CHN 2,500 ppm	3.170[f]	2.687[de]	7.843[c]
CHN 5,000 ppm	2.514[c]	1.757[b]c	7.363[c]
BTH 50 ppm	2.612[c]	2.497[c]d	7.953[de]
BTH 100 ppm	1.403[a]	4.217[g]	10.153[g]
BTH 200 ppm	2.171[b]	3.383[f]	11.227[h]
Control	2.861[d]	0.633[a]	4.053[a]
F-test	**	**	**
CV(%)	22.48	13.46	2.71

[1/]PPO activity was evaluated at 0 and 7 days after treatment with chitosan and BTH or sterile distilled water (control) and at 7 days after challenging with *S. ampelinum* strain GB4 (14 days after treatment); [2/] Each value represents a mean of three replicate plants with two leave per plant. Mean in the column followed by the same letter are not significantly different according to the LSD test (α= 0.05).

Moreover, we found increases in PR proteins and defense enzyme levels in chitosan and BTH-treated plants compared to the non-treated controls, with significant enhancement of these defense compounds being more pronounced in chitosan after pathogen challenging. These results further support the conclusion that chitosan and BTH prime for resistance instead of directly activating it (Aziz et al., 2006; Aziz et al., 2007; Allégre et al., 2009; Dubreuil-Maurizi et al., 2010; Dufour et al., 2012). The significance of priming is that the synthesis of proteins involved in defense occurs only upon pathogen infection, and thus, there are low fitness costs for the plant in the absence of the pathogen (Conrath et al., 2006; Buensanteai et al., 2009; Perazzoli et al., 2008; Legay et al., 2011), as would be expected when treatment with elicitors lead to the direct expression of resistance mechanisms (Heil, 2002; Buensanteai et al., 2009; Körösi et al., 2011).

In conclusion, chitosan and BTH were found to be capable of inducing resistance in grapevine against anthracnose disease and this resistance was associated with the expression of SAR genes. Moreover the activation of SAR biochemical markers, as salicylic acid, PR proteins and defense enzymes, correlated with the resistance degree to anthracnose disease and with disease severity. These findings can have important implications in the use of chitosan and BTH as active-elicitors for integrated plant disease control in vineyard.

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