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Full Length Research Paper

Changes in salicylic acid in grapevine treated with chitosan and BTH against *Sphaceloma ampelinum,* the causal agent of grapevine anthracnose

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Chitosan and BTH selected as inducers of systemic resistance against *Sphaceloma ampelinum,* the causal agent of grapevine anthracnose, are presented for biological control of multiple pathogens causing either foliar or root diseases of various economic crops. In the present study, chitosan and BTH were investigated for their ability to induce some defense related chemicals that protect grapevine from *S. ampelinum* infection. Salicylic acid was found to accumulate in grapevine leaf tissue, treated with chitosan and BTH and challenged by *S. ampelinum* when plants were 60-day-old. The accumulation of salicylic acid (13.08 and 12.15 µg g^{-1} fresh weight) increased for seven days after fungal pathogen challenge inoculation and foliar treatment with chitosan and BTH. In pathogen inoculated plants of non-treated foliar, salicylic acid also accumulated for seven days with very low level of 2.90 µg g^{-1} fresh weight. Moreover, chitosan and BTH reduce anthracnose disease severity up to 75 and 60%, respectively. These results show the potential of using chitosan and BTH to induce resistance of grapevine, and as active-elicitors for plant diseases management. Furthermore, the use of chitosan and BTH may minimize the cost of control strategies and reduce the risk associated with the high use of chemical pesticides in commercial grapevine production in Thailand.

Key words: Anthracnose, grapevine, induced resistance, salicylic acid, *Sphaceloma ampelinum*.

INTRODUCTION

Anthracnose or scab disease of grapevine caused by *Sphaceloma ampelinum* is one of the most serious fungal diseases of grape in tropical area, especially Thailand (Pienpuck et al., 1993; Sompong et al., 2012). This disease appears every year and reduces the quality and quantity of grapes by weakening the vines, causing up to 50% crop losses in many grapevine growing area. In Thailand, grapevine anthracnose disease is most damaging from July to October; it is characterized by small circular to irregular dark brown spots of about 1 to 5 mm in-diameter which later turn gray in the center and dark brown at the margins. The central necrosis tissue often falls off, leaving a shot-hole appearance. Symptoms usually develop in late summer and fall. Optimal condition can defoliate the grape. On berries, the disease is characterized by a typical bird's eye spot which is violet to grayish at the centre and dark brown at the margins of young infected fruit, which generally shrivels and dies throughout the season. On shoots and tendrils, small isolated light brown spot develops which elongates to form elliptical, slightly sunken lesions. Later, the central area of the lesion develops into ashy-gray color bordered by darker rim. The affected shoots may be restricted in

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growth, and shedding of flower buds takes place due to infection (Pienpuck et al., 1993; Sompong et al., 2012).

Anthracnose disease of grapevine in Thailand is normally controlled by fungicide application, but the emergence of new race of the pathogen diversity is reported (Sompong et al., 2012). Moreover, there are more restrictions on fungicide application to the fresh product due to the concern of the public about food safety on human health and environment (Buensanteai et al., 2009). Therefore, the strategy to control the disease is to use induced resistance mechanism (Aziz et al., 2006; Aziz et al., 2007; Trouvelot et al., 2007; Slaughter et al., 2008; Allégre et al., 2009; Godard et al., 2009; Dubreuil-Maurizi et al., 2010; Verhagen et al., 2010; Archana et al., 2011; Iriti et al., 2011; Perazzoli et al., 2011; Verhagen et al., 2011; Hatem et al., 2012).

Induced resistance is a biochemical and physiological state in which active- elicitors such as plant growth promoting rhizobacteria, synthetic chemical and natural products increase plant defense mechanism against various pathogenic infections. Induced resistance studies using chitosan and BTH on cotton, maize, cabbage, sunflower, grapevine and tobacco (Godard et al., 1999; Iriti et al., 2004; Omyma and Mahmoud, 2005; Aziz et al., 2006; Cohen et al., 2011; Korosi et al., 2011; Dufour et al., 2012) revealed that these elicitors can trigger chemical and biochemical mechanisms in plant cell, leading to the resistance of the phenotype. The two most widely studied types of induced resistance are induced systemic resistance (ISR) and systemic acquired resistance (SAR). One is the salicylic acid (SA)-dependent pathway, associated with systemic acquired resistance (SAR) that act on infection caused by many plant pathogens (Sricher et al., 1997; Buensanteai et al., 2009). The second, referred to as the pathogen-induced jasmonic acid (JA)-dependent pathway, was first found to be induced by certain necrotizing plant pathogens via a separate pathway which is SA-independent and JA-dependent (Vidal et al., 1997; Buensanteai et al., 2009). This pathway is distinguished from the SA-dependent pathway in that it leads to the expression of genes for defensin (PDF1.2) and a set of pathogenesis-related (PR) proteins, including a basic *β*-1,3-glucanase (*PR-2*) and a basic chitinase (*PR-3*); whereas the SA-dependent pathway leads to the expression of genes for a different set of PR proteins, most notably *PR-1* (Vidal et al., 1997; Buensanteai et al., 2009)

There is some information regarding traits expressed systemically in grapevine in response to induction by plant growth promoting rhizobacteria (PGPR) and active-elicitors (Hamiduzzaman et al., 2005; Aziz et al., 2006; Aziz et al., 2007; Chong et al., 2008; Slaughter et al., 2008; Trouvelot et al., 2008; Allégre et al., 2009; Godard et al., 2009; Dubreuil-Maurizi et al., 2010; Verhagen et al., 2010; Archana et al., 2011; Iriti et al., 2011; Legay et al., 2011; Perazzolli et al., 2011; Dufour et al., 2012; Hatem et al., 2012). Resistance-related mechanisms expressed in grapevine induced by chitosan and BTH have not been well examined; only an association of anthracnose and other fungal diseases reduction by chitosan oligomers was mentioned (Sathiyabama and Balabramaian, 1995; Awadalla and Mahamoud, 2005; Aziz et al., 2006). Moreover, the acibenzolar-*S*-methyl or benzothiadiazole (BTH) has been reported to be effective against biotrophic pathogens such as *Erysiphe necator* and *Plasmopara viticola,* the causal agent of powdery and downy mildew of grapevine (Dufour et al., 2012).

The aim of this study is to examine grapevine defense response to BTH and chitosan, with emphasis on salicylic acid accumulation, the biochemical marker of resistance, occurring in grapevine with challenge inoculation of *S. ampelinum* pathogen, the causal agent of grapevine anthracnose disease.

MATERIALS AND METHODS

Grapevine plantlets (*Vitis vinifera* cv. Black Queens) were planted at the bottom of individual pots containing water and mixed fertilizer. Grapevine was grown in a green house at 26 ± 2°C, with a photoperiod of 12 h of light and a relative humidity (RH) of 70 ± 10% for 2 months. The plant had one shoots and 7 to 9 leaves. Grapevine plants were watered daily and supplemented with fertilizer every two day.

Elicitor application

Chitosan (low-viscous, Fluka, Germany) and BTH (Bion, 50 WG Syngenta Crop Protection, Basel, Switzerland) were used in different concentrations: 1000, 2500, 5000 ppm for chitosan and 50, 100, 200 ppm for BTH. Two months after planting, all elicitors solutions were applied to upper and lower grapevine leaf surfaces using manual sprayer. Grapevine pots were arranged in RCBD in the greenhouse. The negative control treatment was sprayed only with sterile distilled water.

Pathogen inoculum preparation

S. ampelinum collected from an untreated vineyard in the Suranaree University Technology Farm was isolated on water agar (WA). Mycelia agar was transferred from 3-5 day old *S. ampelinum* grown on WA plates and inoculated onto potato dextrose agar (PDA) plate (potato 200 g/L, dextrose 15 g/L, agar 15 g/L). To obtain conidia, the culture was placed in the dark at 100% RH overnight (Sompong et al., 2012). Conidia were collected from the Petri dish using a loop and then suspended in distilled water. The concentration was adjusted to 10^6 conidia per milliliter using a hemacytometer (Sompong et al., 2012).

Pathogen inoculation and anthracnose disease assessment

Seven days after elicitor treatment, leaves of treated and control grapevine seedling were challenge inoculated by spraying *S. ampelinum* conidia suspension onto the plant. Inoculated grapevine seedlings were kept overnight in the control chamber (Trouvelot et al., 2008; Sompong et al., 2012). Disease severity then was assessed by measuring the grapevine leaf area infection. In each experimental, six grapevine leaves were observed per treatment.

Figure 1. The grapevine anthracnose symptoms; (A) The symptom on leaves; (B) The symptom on berries.

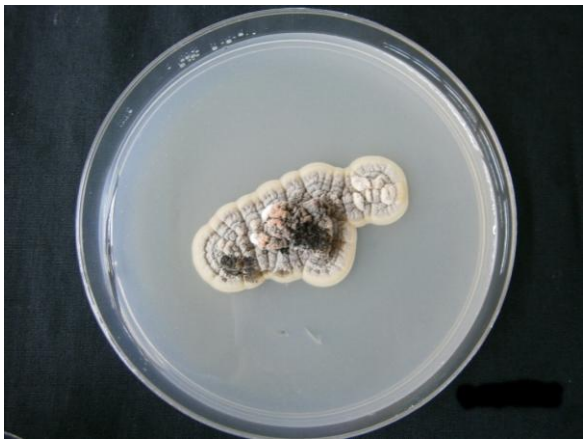

Figure 2. Colony morphology of *S. ampelinum* most virulent strain GB4 on potato dextrose agar (PDA) medium.

For disease severity score, Poolsawat (2008) method was applied: 1 score = leaf area necrosis between 0 to 6%; 2 scores = leaf area necrosis less than 25%; 3 scores = leaf area necrosis between 26 to 50%; 4 scores = leaf area necrosis between 51-75%; and 5 scores = leaf area necrosis over 75% of all leaf area.

Grapevine salicylic acid analysis

To prove that treatment with chitosan and BTH were effective in inducing resistance in the plant experiment, grapevine in the remaining 3 plants per treatment was investigated for salicylic acid accumulation, the biochemical marker of plant resistance. In each plant, at the same development stage, different samples of grapevine leaves were taken: those treated with chitosan, BTH or distilled water. The first sample was collected prior to inoculation with each elicitor, the second sample collected just prior to inoculation with *S. ampelinum* and the other samples collected at the 7 day after *S. ampelinum* pathogen challenge inoculation. The samples were ground in buffer (90 ml methanol: 9 ml acetic acid, 1 ml ddH_2O) with a chilled mortar and pestle; and 0.5 ml of 90% methanol was added to 0.5 g of the obtained sample after being centrifuged at 15,000 xg for 15 min. Supernatant of 500 µl of 0.02 ferric ammonium sulfate was added. The reaction mixtures were inoculated at room temperature for 5 min and absorbance reading at 530 nm. The level of salicylic acid in the grapevine sample was expressed in µg g^{-1}fresh weight. The partial method described by Raskin et al. (1990) and Rasmussen et al. (1991) was slightly modified.

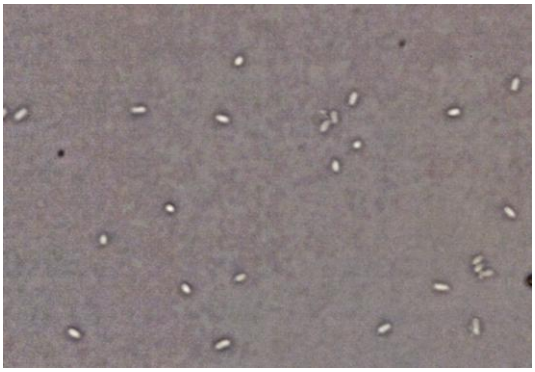

Figure 3. *S. ampelinum* conidia isolated from grapevine cv. Black Queens cultured on potato dextrose agar (PDA) at 30 days (1200x magnification).

Statistical analysis

All experiment was arranged in randomized completely block design with three replicates of 3 plants per treatment and replicated twice per time. For level of SA, analysis of variance (ANOVA) was carried out, and the significance of difference among the treatments was determined according to Duncan's Multiple Range Test at $P < 0.05$ using SPSS version 14.

RESULTS

Sample collection and isolation of *S. ampelinum*

Anthracnose symptoms can be found on all the parts of grapevine plant (Figure 1). In severe case, anthracnose can lead to the complete drying of grapevine leaves, as seen in Figure 1.

The fungal mycelium colony appeared within 3 to 5 day after culture on WA and the colony color on PDA at 35 days was dark brown-black with cotton and flocculent appearance (Figure 2). The virulent conidia were hyaline, single celled, having circular to oblong shape; often conidia are glued together in irregular chains (Figure 3).

Grapevine anthracnose disease assessment

In this experiment, treating grapevine cultivar (Black Queens) with chitosan and BTH reduced the severity of anthracnose in the foliage, confirming the occurrence of induction of systemic resistance. The results indicated that treatment with chitosan and BTH reduced the severity of anthracnose in leaves by 75 and 60%, respectively compared to the sterile distilled water, which is the negative control (Table 1; Figures 4 and 5).

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
CHN 5,000 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 for 7 days after foliar treatment with chitosan and BTH. [2]The disease score included: 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 7 days after challenged with *S. ampelinum* strain GB4 or sterile distilled water. Each value represents a mean of three replicate plants with two leaves per plant. Mean in the column followed by the same letter is not significantly different according to the LSD test (α= 0.05).

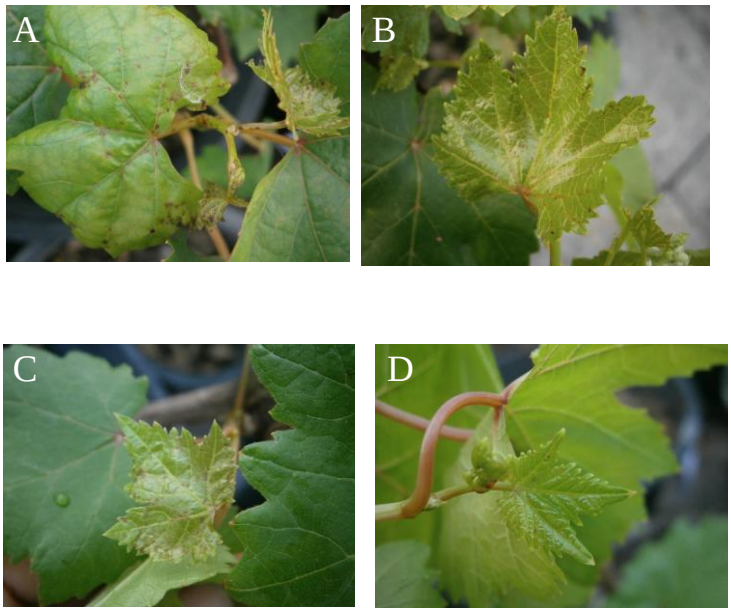

Figure 4. The anthracnose disease symptoms in leaves of grapevine cultivars, Black Queens (A) without or with chitosan concentrations; (B) 1,000 (C) 2,500 and (D) 5,000 ppm.

The level of grapevine salicylic acid

In this study, chitosan and BTH were tested for their ability to induce defense related chemicals to protect grapevine from *S. ampelinum* infection. Grapevine treatment with chitosan and BTH triggered increased accumulation of biochemical marker associated with induced resistance in the presence of *S. ampelinum* pathogen inoculation. In the Black Queens cultivar of grapevine treated with chitosan and BTH, salicylic acid accumulation level was 13.08 and 12.15 µg g^{-1} fresh weight, respectively, after pathogen inoculation, for 7 days (Table 2). In contrast, salicylic acid accumulation in *S. ampelinum* pathogen-inoculated grapevine without chitosan and BTH treatment was considerably lower. We found that in pathogen inoculated plants without foliar treatment, salicylic acid level accumulated for 7 days was very lower (2.90 µg g^{-1} fresh weight).

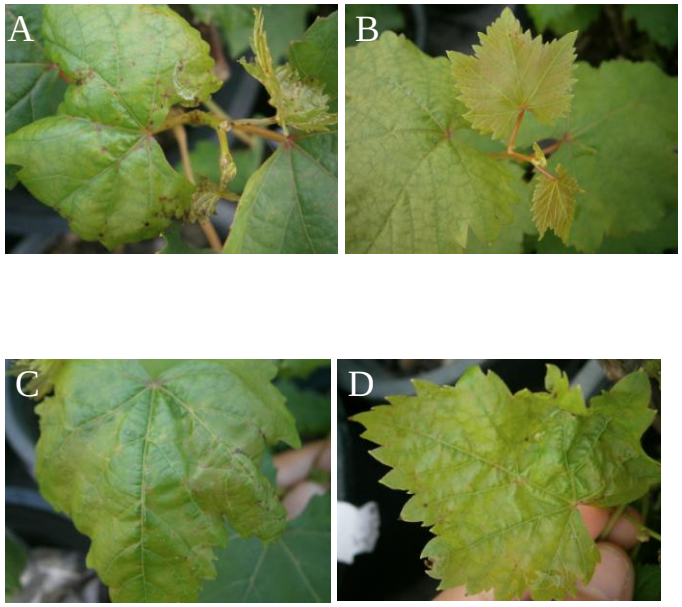

Figure 5. The anthracnose disease symptoms in leaves of grapevine cultivars, Black Queens (A) without or with BTH concentration; (B) 50 (C) 100 and (D) 200 ppm.

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

Treatment	Salicylic acid (µg g^{-1} fresh weight)[1]		
	Time (day)[2]		
	0	7	14
CHN 1,000 ppm	0.78b[3]	3.57e	12.19b
CHN 2,500 ppm	0.60a	3.99f	10.58b
CHN 5,000 ppm	0.99c	2.98d	13.08b
BTH 50 ppm	0.79b	3.06ef	10.85b
BTH 100 ppm	1.04c	3.83ef	12.15b
BTH 200 ppm	1.46e	2.16c	11.54b
Control	1.21d	0.19a	2.90a
F-test	**	**	**
CV(%)	8.63	12.87	18.17

[1]Salicylic acid accumulation was evaluated at 0 and 7 days after treatment with chitosan and BTH or sterile distilled water. And also salicylic acid was determined at 7 days after challenged with *S. ampelinum* strain GB4 or sterile distilled water (14 days). [2]Grapevine leaves were treated with chitosan and BTH or sterile distilled water at 0 day, challenged with *S. ampelinum* strain GB4 or sterile distilled water at 7 days after foliar treatment with chitosan and BTH, and the induced resistance ability at 7 days after challenged with *S. ampelinum* strain GB4 or sterile distilled water was investigated. [3]Each value represents a mean of three replicate plants with two leaves per plant. Mean in the column followed by the same letter is not significantly different according to the LSD test (α= 0.05).

DISCUSSION

In the present experiment, we found that the application of chitosan and BTH through foliar spray can induce resistance in grapevine against anthracnose pathogen, *S. ampelinum.* Our results show that the induction of

resistance by chitosan and BTH in grapevine is due to the concentrations of each elicitor. The most efficient induction was obtained with concentrations of 5,000 ppm chitosan and 200 ppm BTH. Moreover, our study is the first experiment to determine grapevine response to active-elicitor with induced resistance against *S.ampelinum* using analysis of salicylic acid, which is a common plant biochemical response to pathogen. We found increase in salicylic acid accumulation in chitosan and BTH treated grapevine compared to the negative controls, with the elevation of salicylic acid being more pronounced in chitosan and BTH treated grapevine than that inoculated with *S. ampelinum.* The differential expression of the salicylic acid marker after pathogen challenge was similar to the induction of salicylic and jasmonic accumulation in grapevine by β-aminobutyric acid that was potentiated by exposure to a callose formation preparation from *Plasmopara viticol*a, the causal agent of downy mildew (Hamiduzzaman et al., 2005) and is indicative of priming as defined by Sticher et al. (1997) and Buensanteai et al. (2009).

We found increases in SA levels in chitosan and BTH-treated plants compared to the controls, with the elevation of these signaling compounds being more pronounced in chitosan and BTH -treated plants than that inoculated with *S. ampelinum*. These results support our conclusion that chitosan and BTH prime grapevine plants (Buensanteai et al., 2009). They are also evident that chitosan and BTH-induced resistance in grapevine involving JA- and SA- dependent pathways. The latter is likely to be the pathogen-induced SA-dependent pathway because this pathway requires salicylic acid production (Hamiduzzaman et al., 2005; Buensanteai et al., 2009), whereas the rhizobacteria-induced JA-dependent pathway is not associated with increased production of JA (Buensanteai et al., 2009). The pattern of differential accumulation of salicylic acid in *S. ampelinum*-inoculated and non-inoculated grapevine pretreated with chitosan and BTH further supports the occurrence of priming. Priming appeared to occur only in genotypes with intact SA or JA or ethylene sensitivity, thus indicating that induction of priming by chitosan and BTH required activation of a SA-dependent pathway in grape plant, and which is consistent with chitosan and BTH induced resistance to *S. ampelinum*. Salicylic acid production also was reported to be associated with priming induced by thiamine and BABA, but SA-dependent signaling was required (Malamy et al., 1990; Hamiduzzaman et al., 2005; Eschen-Lippuld et al., 2010). In addition, the induction of biological maker as hydrogen peroxide as the intermediate of salicylic acid in barley using the chemical elicitor DCINA was SA independent. Therefore, the involvement of JA- and SA-dependent pathways in the induction of salicylic acid varies depending on the elicitor-host plant interaction.

Based on the response of grapevine to chitosan and BTH, we can surmise that chitosan and BTH activate a SA-dependent pathway in this crop. While this pathway can lead to enhanced resistance against broad spectrum of the pathogens, the resistance mechanisms may occur. In addition, SA signaling is also involved in induced resistance against herbivorous insects. Studies have shown that combinations of active-elicitors that induce complementary pathways can result in the induction of a higher level or broader range of resistance (Buensanteai et al., 2009; Mandal et al., 2009; Manjunatha et al., 2009). The apparent activation of both pathways in grapevine by chitosan and BTH, coupled with the ability of the active-elicitor to induce plant defense against fungal pathogens via induced resistance mechanism (Friedrich et al., 1996; Eikemo et al., 2003; Buensanteai et al., 2009) suggest that application of this single elicitor to grapevine could potentially provide protection against a broad spectrum of plant pathogens and, perhaps, also insects. We demonstrated that treatment with chitosan and BTH results in priming of plants against pathogen infection. The significance of priming is that production of proteins important in defense is mostly held in check until needed, that is, upon pathogen infection; and thus, there is little cost in yield lost to the priming process in the absence of pathogens (Buensanteai et al., 2009; Perazzoli et al., 2008), as would be expected when treatment with an inducing elicitor leads directly to the expression of resistance mechanisms (Buensanteai et al., 2009).

In conclusion, chitosan and BTH were found to be capable of inducing resistance in grapevine against anthracnose disease. During the induced resistance reactions, the accumulation of salicylic acid was increased. Moreover, activation of this biochemical marker as salicylic acid did correlate with the degree of anthracnose disease resistance and disease symptoms. The relationship of disease symptom and level of salicylic acid occurs when the elicitor treatment could change and increase level of salicylic acid because it is the important process of signaling that induces resistance process in plant. The finding from this study sheds new light on the interaction of grapevine with active-elicitor such as chitosan and BTH. Our findings do have important implications for the use of chitosan and BTH as active-elicitors for the new strategy of controlling grapevine disease in Thailand.

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