

THE APPEARANCE OF SYSTEMIC ACQUIRED RESISTANCE COMPONENTS IN SA-TREATED OR NATURALLY INFECTED *VITIS* PLANTS BY *UNCINULA NECATOR*

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Abstract

In the present study salicylic acid (SA) treatment and challenge by *Uncinula necator* (Schwein.) Burr., significantly induced the systemic acquired resistance components in grapevine (*Vitis vinifera* L. cv. Sultana). Chitinase (CHV, EC 3.2.1.14) and β -1,3-glucanase (Laminarinase, EC 3.2.1.39) activities increased in SA-treated (100, 200 and 400 ppm) as well as in *U. necator* infected plants and the highest enzymatic activity was observed in leaves treated with 100 ppm of SA. In contrast, total protein content and accumulation of endogenous free-SA in the leaves were observed at the highest rate of 200 ppm of SA-treatment. Salicylic acid also affected photosynthetic pigments by causing decrease in chlorophyll *a* and *b* contents and increase in carotenoids content. Treatment of grapevine leaves with SA provides an attractive tool for the control of powdery mildew disease in grapevine.

Introduction

Plants have evolved complex mechanisms to defend themselves against pathogens, and a great deal of attention has been directed towards elucidating the molecular nature of such mechanisms (Durner *et al.*, 1997). Recent advances in research on plant defence signalling pathways have shown that plants activate defence pathways that vary depending on the type of invader encountered (van Wees *et al.*, 2000). Salicylic acid (SA) has been recognized as a signalling molecule involved both in local defence reactions at infection sites and in the induction of systemic acquired resistance (SAR) (Durner *et al.*, 1997). The synthesis and accumulation of SA are thus important requirements for a defence response.

Moreover, SAR is usually accompanied by synthesis of pathogenesis-related proteins (PR- proteins) (Linthorst, 1991). It has been reported that some PR-proteins have chitinase (PR-2 group) and β -1,3-glucanase (PR-3 group) activity *in vitro* (Mauch *et al.*, 1988; Arlorio *et al.*, 1992). These hydrolytic enzymes capable of degrading fungal cell wall polysaccharides, chitin and β -1,3-glucans could thus inhibit fungal growth (Roulin & Buchala, 1995). Enhanced resistance to fungal pathogens has also been found in transgenic plants over expressing chitinase or β -1,3-glucanase, with synergistic benefit where both genes are present (Jach *et al.*, 1995; Jongedijk *et al.*, 1995). The application of SA to tobacco induced PR gene expression and enhanced resistance to pathogens such as tobacco mosaic virus (TMV) (Durner *et al.*, 1997). Elicitations with SA or with strains of *Botrytis cinerea* induced several PRs extractable at pH 2.8 which were found to accumulate in grapevine leaves (Derckel *et al.*, 1998).

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Grapevine is a crop of world-wide economic importance which is prone to several devastating fungal diseases which requires frequent application of fungicides (Agrios, 1997). One of these devastating diseases is powdery mildew, caused by *Uncinula necator* (Schwein.) Burr., whose control often requires heavy application of appropriate fungicides. Induced resistance could then provide systemic protection against infection to substitute for or supplement control by standard fungicides.

Our limited information about inducible defence mechanism in grapevines, to date has tried to determine focusing on the induction of a number of PR proteins, including chitinases and β -1,3-glucanases in grapevine leaves following the application of SA or infection by pathogens (Renault *et al.*, 1996; Repka, 2001). Busam *et al.*, (1997) reported differential expression of two chitinase genes in grapevine responding to SAR activators and fungal challenge with *Plasmopara viticola*. More recently, Giannakis *et al.*, (1998) reported a correlation between the combined activities of chitinase and β -1,3-glucanase of a range of grapevine cultivars and their observed field resistance to powdery mildew. In present study we examined the effectiveness of SA treatment and *Uncinula necator* infection on the changes of activities of chitinase and β -1,3-glucanase and endogenous-free SA level, which are the main components of SAR.

Materials and Methods

Plant material and field trials: Field experiments were conducted for two consecutive years in two different vineyards, 9-year-old and located at Akcapinar (Salihli-Manisa), on the eastern coast of Aegean Sea. Test plots (2 plots/vineyard; 100 plants/plot) were arranged in a completely randomized block design with four replicates (25 plants each). One vineyard had *U. necator* infection on leaves at the level of 48% and the other vineyard was free of infection. Leaves of 3 groups of plants were studied: plants sprayed with distilled water (negative control); plants sprayed with SA (100, 200 and 400 ppm), plants naturally infected with *U. necator*.

SA treatment: Different concentrations of SA (100, 200 and 400 ppm) were prepared from a stock solution of 1 mg SA ml⁻¹ distilled water with the addition of 0.1% Tween 80. Grapevine plants were treated by spraying to run-off with SA solution or distilled water as a control. Mature and about same size leaves were harvested 3 days after with SA treatment and frozen in liquid nitrogen and stored at -20°C for analyses.

Preparation of leaf homogenates and partial purification of proteins: Leaf homogenates were obtained by grinding leaves (5 g) in a mortar containing 0.1 M Sodium acetate (NaAc) buffer (pH 5.0, 2.0 ml g⁻¹ fresh weight). Homogenates were centrifuged for 15 min at 9 000 g. Proteins were precipitated by Ammonium sulphate [(NH₄)₂SO₄], 80% saturation (51.6 g in 100 ml). The pellet obtained after centrifugation (5 min 1 000 g) was air dried, suspended in 0.1 M NaAc buffer (pH 5.0), dialyzed against NaAc buffer (pH 5.0) and used for enzyme assays and protein determination. The protein contents were determined by the dye binding method of Bradford (1976), with bovine serum albumin (Sigma, Dorset, UK) as a standard.

Chitinase assay: Chitinase activity was determined by the method of Hackman & Goldberg (1964). Chitinazur (10 mg) (Sigma) was used as substrate for this enzyme assay and was mixed with 1.5 ml of enzyme solution for 24 h at 37°C. After centrifugation at 9 000 g the absorbance of the supernatant was determined at 575 nm in spectrophotometer (Unicam α , Helios, Berlin, Germany).

β -1,3-Glucanase assay: β -1,3-glucanase activity was assayed by measuring the rate of reduction in sugar production with laminarin (Sigma) as the substrate. The reaction mixture consisted of 0.1M NaAc buffer (pH 5.5) containing 1% reduced laminarin and enzyme solution. After 30 min of incubation at 37°C, and cooling on ice, alkaline copper reagent and 0.12% fresh neocuproine solution were added. The mixture was heated at 100°C for 10 min. After returning the tubes to the ice-bath, 1.5 ml of H₂O was added. The absorbance was measured at 450 nm (Boller, 1992) using glucose as a standard.

SA determination: Extract for the determination of free-SA was prepared following the procedure of Raskin *et al.*, (1989). Control leaves, SA-treated leaf samples and infected leaves (1 g) were ground with an aqueous solution of 90% (v:v) methanol and centrifuged at 9 000 g for 30 min. The supernatant was dried under low pressure at 40°C. The residue was resuspended in 5% Trichloro-acetic acid and centrifuged for 10 min., at 1 200 g. The supernatant was partitioned with a 5 ml 1:1 (v:v) mixture of Ethyl acetate/hexane containing 1% (v:v) isopropanol. The top organic phase was dried under low pressure at 40 °C and resuspended in 0.5 ml of the HPLC mobile phase [45% water and 55% methanol with 0.025% (w:v) H₃PO₄]. HPLC separation of SA was performed by Hewlett Packard (Hewlett Packard, model 1100, Palo Alto, CA, USA) equipped with a Nucleosil C18 column (150 x 4 mm). The column was maintained at 40°C with a mobile phase flow rate of 1 ml dk⁻¹. Salicylic acid concentration in 0.1 ml sample was determined by an HPLC diode array detector at 302 nm. Identification of free SA was made by the retention time and coincidence with the pure standard purchased from Sigma (Dorset, UK). The quantification of salicylic acid was obtained by using calibration curve of the pure SA standard. The lowest concentration of salicylic acid reliably quantifiable by the procedure was 0.01 µg g⁻¹ of fresh weight. The procedure had a 55% recovery rate for extractable salicylic acid as determined by tissue samples with salicylic acid.

Measurement of photosynthetic pigments and total soluble solids: The photosynthetic pigments (chlorophyll *a*, *b* and carotenoids) of grapevine leaves (0.1 g) were measured by the method of Witham *et al.*, (1971) using 80% acetone.

Ripe grapes were collected and total soluble solids (°Brix), mainly represented by glucose and fructose (Salzman *et al.*, 1998), were measured with a hand-held refractometer (Model 10430, Reichert, Vienna, Austria). The molarities of total soluble solids in the expressed juice were calculated from refractive index measurements using a glucose standard curve.

Statistical analysis: All data were subjected to one-way ANOVA test with use of statistical software of SPSS 6.1, and means were compared by the protected least significant difference (LSD) test. Comparisons with *p* values less than 0.05 were considered significantly different.

Results

Changes in total protein content: SA treatment and *U. necator* infection caused an increase in the total protein content of leaves (Fig. 1). The highest protein content was detected in infected leaves, when there was a 3.5-fold increase in protein content of the leaves compared with the controls. After SA treatment, protein content of leaves reached 0.53 mg ml⁻¹ (2.5-fold) at 200 ppm. SA at 100 ppm and 400 ppm caused 1.9- and 0.8-fold increase respectively over the control.

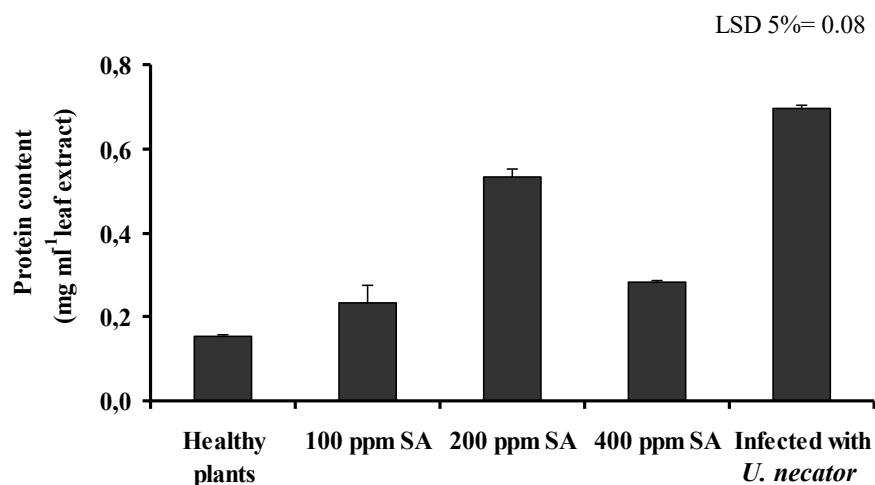


Fig. 1. Total protein contents (mg ml⁻¹) of healthy plants as control, SA-treated leaves and *U. necator* infected leaves of *Vitis vinifera* L. cv. Sultana. Vertical lines on the graph denote the SE.

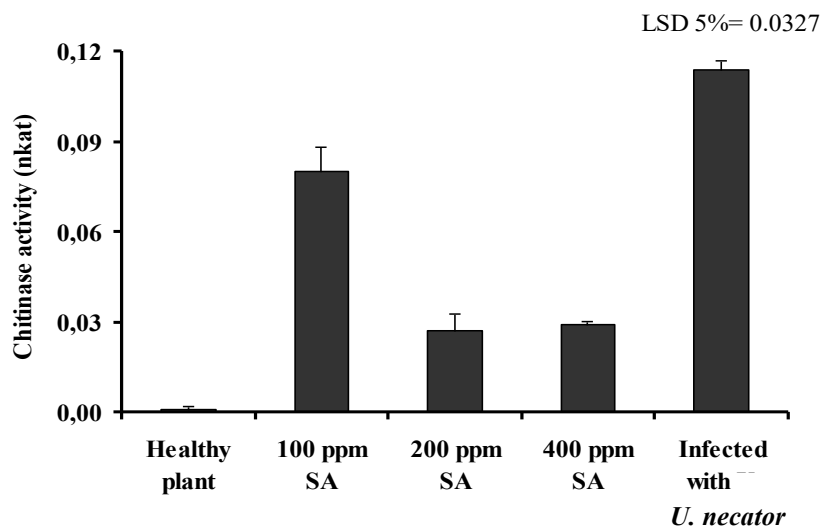


Fig. 2. Chitinase activity of leaves of healthy plants as control, SA-treated leaves and *U. necator* infected leaves of *Vitis vinifera* L. cv. Sultana. Vertical lines on the graph denote the SE.

Changes in chitinase and β -1,3-glucanase activity: Chitinase activity of 100 ppm SA-treated grape leaves increased about 78-fold comparing the control (Fig. 2). The two higher SA concentrations (200 and 400 ppm) and *U. necator* infection also caused a significant increase in chitinase activity. β -1,3-glucanase activity of homogenates of SA-treated leaves and of leaves infected with *U. necator* increased significantly and peaked in leaves treated with 100 ppm SA as much as 93% (Fig. 3). SA at 200 ppm showed almost close results of glucanase activity with the 78% changes in the activity. Although there was the lowest activity at 400 ppm SA-treated leaves, it was at significant level compared with control.

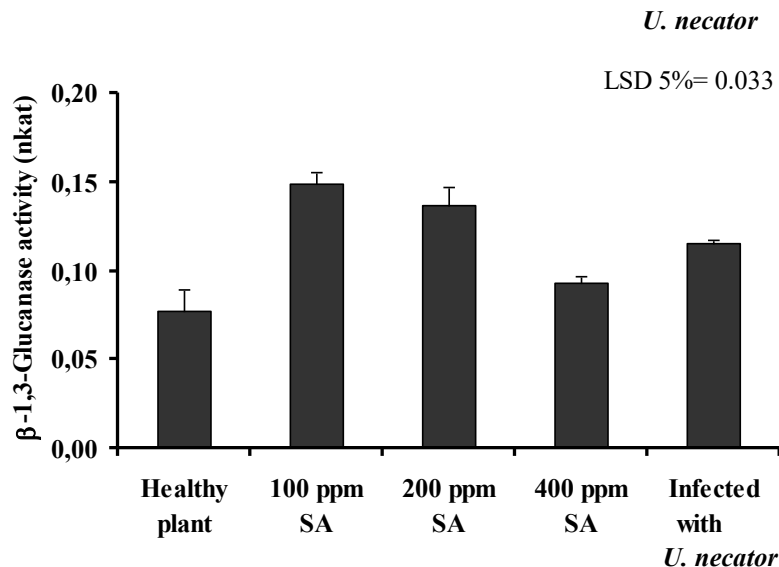


Fig. 3. β-1,3-Glucanase activity of leaves of healthy plants as control, SA-treated leaves and *U. necator* infected leaves of *Vitis vinifera* L. cv. Sultana. Vertical lines on the graph denote the SE.

Table 1. Effect of SA treatment and *U. necator* infection on photosynthetic pigment contents in grapevine leaves. Data are the average and $\pm$ standard error of 4 replicates. Chl, chlorophyll.

Treatment	Photosynthetic pigment content (mg g ⁻¹ fresh weight of leaves)			
	Chl <i>a</i>	Chl <i>b</i>	Chl <i>a/b</i>	Carotenoid
Healthy plants	1.081 $\pm$ 0.005	0.639 $\pm$ 0.009	1.691 $\pm$ 0.018	2.887 $\pm$ 0.433
100 ppm SA	0.900 $\pm$ 0.022 *	0.479 $\pm$ 0.010 *	1.878 $\pm$ 0.023 *	4.072 $\pm$ 0.083 *
200 ppm SA	1.086 $\pm$ 0.144	0.578 $\pm$ 0.023	1.869 $\pm$ 0.101 *	5.257 $\pm$ 0.281 *
400 ppm SA	0.742 $\pm$ 0.095 *	0.395 $\pm$ 0.017 *	1.871 $\pm$ 0.081 *	3.575 $\pm$ 0.320 *
Infected with <i>U. necator</i>	0.863 $\pm$ 0.069 *	0.478 $\pm$ 0.035 *	1.799 $\pm$ 0.011 *	3.808 $\pm$ 0.364 *
	LSD 5%= 0.181	LSD 5%= 0.16	LSD 5%= 0.108	LSD 5%= 0.688

* $p < 0.05$ as compared to a different SA treatment with the LSD test.

Changes in endogenous free-SA content: The free-SA content of leaves increased significantly by SA treatment and *U. necator* infection (Fig. 4). The highest SA level was measured at 200 ppm SA treatment, when it reached to 0.64 $\mu\text{g g}^{-1}$ fresh weight, about 4.84-fold increase over the controls. SA-treated leaves had more free-SA content than infected leaves. Leaves treated with 100 ppm SA showed 1.54-fold increase and 400 ppm SA-treated leaves had 1.09-fold increases in endogenous free-SA accumulation. *U. necator* infection led to 72% elevation in free-SA content of leaves.

Photosynthetic pigment contents of and total soluble solids: SA treatment and powdery mildew disease significantly reduced the concentrations of photosynthetic pigment (chlorophyll *a*, *b*) contents in the leaves (Table 1). Chlorophyll *a* and *b* content did not change in leaves treated with 200 ppm SA compared with control. In contrast to chlorophyll *a* and *b*, carotenoids content of leaves significantly increased in all SA-treated and infected leaves over the controls. The total soluble solids content of grape berries was not affected by SA treatment to leaves ($p < 0.05$).

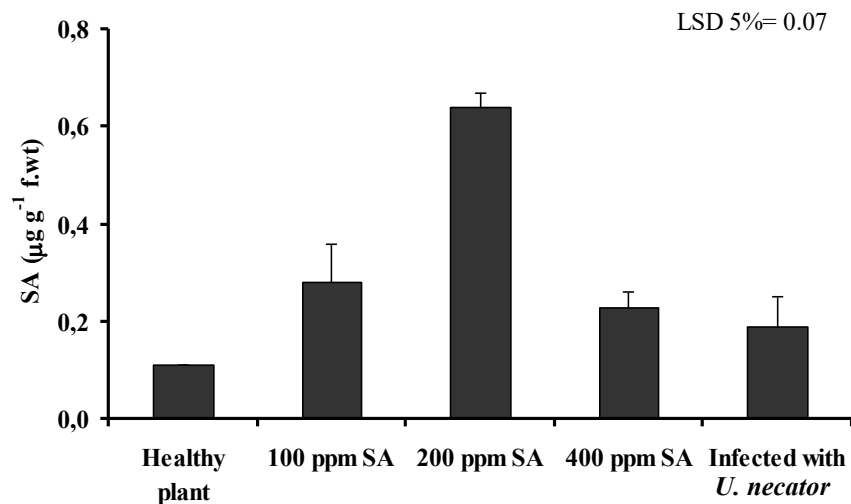


Fig. 4. Endogenous salicylic acid contents ($\mu\text{g g}^{-1}$ fresh weight) in leaves of healthy plants as control, SA-treated leaves and *U. necator* infected leaves of *Vitis vinifera* L. cv. Sultana. Vertical lines on the graph denote the SE.

Discussion

Several research works demonstrated that SA plays a key role in the signal transduction pathway leading to the establishment of SAR and that exogenous application of SA may induce resistance to a member of pathogens which attack many cultivated plants (White, 1979; Metraux *et al.*, 1990; Metraux & Raskin, 1993; Gaffney *et al.*, 1993; Quintanilla & Brishammar, 1998; Qin *et al.*, 2003). In case of grapevine, it has been observed that grapevine leaves were protected from powdery mildew by pre-treatment with SA. The results of our work seem to confirm those of Busam *et al.*, (1997), who reported inhibitory effect of SA against *Botrytis cinerea* conidia in grapevine.

In the study we found that SA treatment and *U. necator* infection caused free-SA accumulation in the grapevine leaves. Both biotic and abiotic factors have the potential to activate the SA biosynthesis and accumulation is well known (Enyedi, 1999; Durner *et al.*, 1997), in tobacco and cucumber, this accumulation paralleled transcriptional activation PR genes (Ryals *et al.*, 1996). This works and earlier observations of Schneider-Muller *et al.*, (1994) reported rapid synthesis of SA on carrot by fungal elicitation which supports our findings that SA accumulation is necessary for appearance of SAR.

Besides the enhanced SA levels, findings about the greater activity of intercellular chitinase and glucanase in the grapevine leaves suggested that SA acted as an endogenous signal for the plant activating host resistance to powdery mildew. Our results corroborate those of Metraux *et al.*, (1989), who reported chitinase accumulation in SA-treated leaves in cucumber and the findings of Bokshi *et al.*, (2003) explaining elevated activity of β -1,3-glucanase as an indicator of the phenomenon of chemically induced resistance. In addition, a similar results were obtained in perennial woody plants, *Magnifera indica* and *Pyrus bretschneideri* (Zeng *et al.*, 2006; Cao *et al.*, 2006). In grapevine leaves treated with SA and *U. necator* showed an increase in the total protein level, and in chitinase and β -1,3-glucanase activity. Enhanced chitinase activity was

evident 3 days after SA treatment. At this time there were no macroscopic mildew symptoms on the grapevine leaves. This indicated that the SA solution caused the plant to respond rapidly to pathogen attack by synthesizing chitinases and glucanases. In addition, *U. necator* infection of leaves lead to raise both total protein content and enzyme activities. An analogical situation was reported for grape berries cv. Pinot Noir with SA increased the level of chitinase, and that of β -1,3-glucanase (Busam *et al.*, 1997). Our results indicating a correlation between resistance rating and activity of chitinase and β -1,3-glucanase of the leaves in grapevine are strongly consistent with the findings of Giannakis *et al.*, (1998) who clearly demonstrated the associations between SAR and chitinase and β -1,3-glucanase activities by screening 21 different grapevine genotypes with varying resistance to powdery mildew.

Like protein metabolism, photosynthetic metabolism was affected by *U. necator* infection or SA treatment. Clearly, the plant pathogens reduced photosynthesis, by acting upon the chloroplasts and causing their degeneration (Agrios, 1997). Although SA treatment compensated for the negative effects of pathogens on protein metabolism, it does not act in the same manner in photosynthetic metabolism. Interestingly, a decrease in chlorophyll *a* and *b* contents in 100 and 400 ppm SA treatment may be accompanied with senescence. SA was reported to be capable of inhibiting of ethylene biosynthesis and reversing ABA-induced some physiological responses (Malamy & Klessig, 1992; Raskin, 1992), our results showed that SA may have a role in senescence as a plant hormone. These results support the findings of Krupinska *et al.*, (2002) who found a connection between the nuclear proteins encoded HvS40 which is induced by jasmonate and salicylic acid and the degeneration of chloroplasts occurring during senescence and during infection of barley with *Pyrenophora teres*. In the current research, unlike 100 and 400 ppm SA, 200 ppm SA treated leaves did not showed significant change in chlorophyll *a* and *b* contents and that was dose of the highest free-SA accumulated treatments in leaves. This result is in agreement with previous studies which report that SA did not cause chlorophyll loss in dark treated barley seedlings (Popova *et al.*, 2003) and cowpea (Chandra & Bhatt, 1998).

Biotic stress or activation of the resistance mechanism by SA treatment leads to increase in carotenoid pigments contents like environmental stress. These similar effects of both factor indicated that plants defence themselves triggering carotenoids biosynthesis in leaves. This pigment group had no direct role in plant defence system. Their activities were increased by high hydrogen peroxide content in cells (Tatsuzawa *et al.*, 2000; Arnao *et al.*, 2001), thereby catalase inhibition was caused by SA treatment or hypersensitive response-associated oxidative burst (Durner *et al.*, 1997).

In conclusion, foliar SA sprays may provide remarkable protection against powdery mildew disease of *Vitis vinifera* L. cv. Sultana in practice by triggering systemic acquired resistance components. Our results suggest that SA treatment holds promise in substituting for fungicidal control of powdery mildew disease in grapevine.

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