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ORIGINAL PAPER

Modifications of antioxidant activity and protein composition of bean leaf due to *Bean yellow mosaic virus* infection and salicylic acid treatments

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Abstract The antioxidant status as well as protein composition of faba bean leaves infected with *Bean yellow mosaic virus* (BYMV) and the effect of salicylic acid application was examined in this paper. Some modifications in the antioxidant status were observed by changing some antioxidant enzymes activities and contents of antioxidant metabolites. BYMV-infected leaves revealed POD, CAT, APX and SOD induced activities while SA treatments could inhibit POD, CAT activities but induced SOD activity. The enzyme activities seemed to be SA concentration dependant. Higher H_2O_2 and MDA concentrations were recorded in virus-infected leaves than those of the corresponding controls while treatment with SA followed by virus inoculation caused lowering of MDA concentration and reducing the damage due to lipid peroxidation. Moreover, because of virus infection and/or SA treatments, an increase in the amounts of phenolics and flavonoids was noticed. As compared to the control, BYMV infection or SA application caused pronounced increase in the antioxidant activity of leaf extracts detected by DPPH assay, indicating an increase in the amounts of antioxidant compounds occurred. To test the protein composition, the contents of each protein fractions (soluble, insoluble and total) were analyzed and the change in protein patterns was visualized using SDS-PAGE. The BYMV-infected bean leaves had protein contents higher than the control indicating accumulation of pathogenesis-related proteins. Moreover, spraying SA with or without virus inoculation could accumulate soluble, insoluble and total proteins and the pattern of increase was in accordance with SA concentration. Alterations in protein patterns were observed in faba bean leaves (*Vicia faba* cv Giza 461) in response to BYMV infection and SA treatments. Because of BYMV infection and SA treatments, the protein profiles showed new bands in comparison to the control. Some polypeptides were highly accumulated in treatments of SA followed by virus inoculation. Changing antioxidant status and accumulation of some antioxidant metabolites as well as the pronounced alterations in the protein composition indicate a kind of plant response against pathogen invasion and in case of SA treatment it is considered a way by which a defence response was initiated and/or activated.

Keywords Antioxidant capacity · *Bean yellow mosaic virus* · Faba bean · Protein content · Protein patterns · Salicylic acid

Abbreviations

AOS	Active oxygen species
APX	Ascorbate peroxidase
CAT	Catalase
POD	Peroxidase
SOD	Superoxide dismutase
SA	Salicylic
DPPH	1,1-Diphenyl-2-picrylhydrazyl

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MDA Malondialdehyde
BYMV *Bean yellow mosaic virus*

Introduction

Active oxygen species (AOS) accumulation causes oxidative damage leading to the appearance of epidemiological symptoms in virus-diseased plants. AOS can oxidize organic compounds and induce membrane lipid peroxidation in the cellular environment (Schützendübel and Polle 2002). Due to their highly reactive nature, AOS are kept as low as possible in plant cells by an efficient AOS-scavenging system, including enzymatic antioxidants (Mittler 2002) and metabolites (Mittler et al. 2004; Foyer and Noctor 2005).

The main antioxidant enzymes are superoxide dismutase (SOD) catalyzing the dismutation of O_2^- to H_2O_2 and O_2, catalase (CAT) that dismutates H_2O_2 to oxygen and water, and ascorbate peroxidase (APX) that reduces H_2O_2 to water by utilizing ascorbate as specific electron donor. These enzymatic systems function for protecting plant cells against oxidative damage (Cagno et al. 2001; Noctor and Foyer 1998; Tommasi et al. 2001). Moreover, other enzymes involved in the ascorbate–glutathione ASC–GSH cycle [glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR)] as well as glutathione peroxidase (GPX) and glutathione-*S*-transferases, are important in protecting cells against oxidative stress.

It was reported that significant changes in the activities of the antioxidant enzymes were previously detected in response to virus infection (Radwan et al. 2006). Peroxidase shows increase in its activity in *Cucurbita pepo* plants infected with *Cucumber mosaic virus* (CMV) (Técsi et al. 1996) and *Zucchini yellow mosaic virus* (ZYMV) (Radwan et al. 2006, 2007). SOD activity declines following the infection of resistant soybean with soybean mosaic virus (Zhuang et al. 1993). Catalase (CAT) activity has been shown to decline following virus infection of a susceptible host and this is believed to lead to increased H_2O_2 levels, which might in turn lead to the induction of systemic acquired resistance (Chen et al. 1993, 1995). Clarke et al. (2002), reported enhancement of peroxidase activity while catalase, glutathione reductase and SOD rapidly declined after infection of *Phaseolus vulgaris* with WCIMV. The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of superoxides and H_2O_2.

H_2O_2 is considered a major compound produced during the oxidative burst in plants. It is the most long-lived and capable of crossing plant cell membranes and thereby act as diffusible and relatively lasting signal. Since the mode of SA signaling pathway is associated with increased H_2O_2 levels (Chen et al. 1993), H_2O_2 has been considered as an essential signal involved in plant defense against biotic and abiotic stressors (Mittler et al. 2004; Foyer and Noctor 2005). It was supposed that the increased H_2O_2 induced by SA may act as an important secondary messenger to improve plant defense mechanisms. SA has identified regulatory roles in the metabolism of plants (Popova et al. 2003). H_2O_2 accumulation may include the induction of at least one of the cellular protective mechanisms (Radwan et al. 2008) that are concomitant with the accumulation of AOS suggesting that SA application results in a state of oxidative stress (Mittler 2002).

One of the best-known lipid peroxidation products, malondialdehyde (MDA, alternatively called 'malonaldehyde') is produced during oxidative burst within the plants (Esterbauer et al. 1991; Marnett 2002). MDA is produced in plants exposed to adverse environmental conditions and is a reliable indicator of free radical formation in the tissue (Parida et al. 2004). Estimation of malondialdehyde (MDA) amount, which is a secondary end product of polyunsaturated fatty acid oxidation, is widely used to measure the extent of lipid peroxidation as indicator of oxidative stress and membrane damage (Lin and Kao 2000; Chaoui et al. 1997). It was reported that accumulation of MDA and H_2O_2 was caused by abiotic stress factors such as salinity and drought (Mukherjee and Choudhuri 1983; Sairam and Srivastava 2002; Radić et al. 2006), heavy metals (Pál et al. 2005) and some biotic stress factors such as virus infection (Beleid El-Moshaty et al. 1993). Moreover, Radwan et al. (2006), reported formation of elevated amounts of MDA and H_2O_2 in *C. pepo* leaves indicating lipid peroxidation and oxidative stress in response to ZYMV infection.

Phenolic compounds are secondary metabolites in plant cells, synthesized through the shikimate pathway or liberated from storage sources and they accumulate in response to avirulent pathogens (Enyedi et al. 1992; Herrmann and Weaver 1999; Chong et al. 2002; Gachon et al. 2004). Specific phenolic compounds in different plant–pathogen interactions could open the way not only for revealing a pathogen attack but possibly also be integrated with an automatic plant stress resistance screening programs (Chaerle et al. 2007). Recently, it was reported that the higher total phenolic content of the plant resulted in higher total antioxidant capacity (Cai et al. 2004). Formation of phenolic compounds is induced in plants pretreated with SA (Guleria et al. 2005). Ali et al. (2007) showed that SA induces the accumulation of phenolic compounds in ginseng root by altering the

activities of enzymes involved in phenolic compounds synthesis. Flavonoids, a group of phenolic compounds, have antioxidant properties inside the plant cells. It should be noted that polyphenolic "flavonoids" from plant sources possess antimicrobial properties (Cushnie and Lamb 2005). The mechanism of antiviral action of polyphenolic compounds such as flavonoids is based on their abilities to act as antioxidants, inhibit certain enzymes, disrupt cell membranes, prevent viral binding and penetration into cells, and trigger a host cell self-defense mechanisms (Friedman 2007).

Pathogenesis-related proteins (PRPs) are well known to be induced in plant tissues in response to pathogen infections, especially during the hypersensitive and systemic response (Van Loon et al. 1994; Van Loon and Van Strien 1999). The PRPs family functions in a wide range from cell wall rigidification to signal transduction and antimicrobial activity (Christensen et al. 2002; Van Loon et al. 2006). However, the biological and biochemical functions of these PRPs during the defense reactions and developmental processes are unclear. The plants infected with a strong isolate of *Potato virus X* had higher total content of PR proteins than healthy plants and those infected with weak isolates (Sapotsky et al. 2005). The accumulation of PR proteins in sensitive plants infected with viruses was reported by other authors (van Loon and van Kammen 1970; Fraser 1982). Islam et al. (2003) noticed increased total nitrogen and protein contents associated with reduction in sugar contents in tomato fruits infected with *Tomato yellow leaf curl virus*. In addition, soluble proteins content of leaf extracts decreased significantly in tobacco after PVY infection (Ryšlavá et al. 2003).

Recent studies have shown that SA also plays an important role in plant resistance to various biotics such as virus infection (Borsani et al. 2001; Radwan et al. 2006, 2007, 2008). SA can provide tolerance against other stresses such as drought and freezing tolerance of winter wheat (*Triticum aestivum*) (Horváth et al. 2007), paraquat-induced oxidative damage in barley (Ananievaa et al. 2004) and heavy metal stresses (Metwally et al. 2003; Yang et al. 2003; Drazic and Mihailovic 2005). SA may also have a role in the defence against ozone stress, as demonstrated in *Arabidopsis thaliana* (Sharma et al. 1996).

The present work is to monitor the changes in the antioxidant status, H_2O_2 contents as well as protein composition of bean leaves under virus infection and SA treatments. It is also to proof a SA-dependant mechanism of induced resistance working against BYMV in bean plants. These mechanisms were suggested to occur through accumulation of H_2O_2 and phenolics or modifications of antioxidant capacity.

Materials and methods

Plant materials and treatments

Seeds of faba bean, *Fabaceae* (*Vicia faba* cv. Giza 461) were sown in a mixture of sand and clay (1:2 v/v) in plastic pots (30 cm in diameter) in natural and favorable conditions suitable for bean growth. The relative humidity was about 70%. The plants were kept at 100% water holding capacity. BYMV used in these experiments was prepared from fresh severely infected leaves of faba bean.

After 21 days of growth, plants with similar size were selected and divided into groups. Each group consists of four replicates (a replicate is one pot containing three healthy plants). The identification of the groups was as follows:

- Control, sprayed with water.
- Infected, inoculated with virus at the same time with the other groups.
- 10, 50 and100 μM (SA + BYMV), sprayed by SA and inoculated with virus 3 days later (SA + BYMV).
- 100 μM SA + BYMV Mix, SA was mixed with the prepared virus inoculum (1:1 v/v) and inoculated.
- 100 μM SA, sprayed without followed virus inoculation.

SA was sprayed to the foliage till run off and then inoculated with virus 3 days after SA spraying.

Antioxidant system measurements

Peroxidase activity

Peroxidase (POD, EC 1. 11. 1. 7) activity was determined by homogenizing a known weight of the fresh leaves material at 4°C in the extraction buffer of 50 mM phosphate buffer pH 7.0 (1:1 w/v). The homogenate was centrifuged at 15,000 rpm at 4°C for 15 min. To measure POD activity, 50 μl of the supernatant was mixed with 10 ml of reaction mixture and spectrophotometrically measured. The assay mixture contained 50 mM potassium phosphate pH 7.0, 0.1 mM EDTA, 5 mM guaiacol and 0.3 mM hydrogen peroxide. Increase in the absorbance due to oxidation of guaiacol (extinction coefficient = 26.2 mM cm^{-1}) was measured at 470 nm. Enzyme activity was calculated in terms of μmol of guaiacol oxidized min^{-1} g^{-1} fresh weight at 25 ± 2°C (Mac Adams et al. 1992; Zhang 1992).

Ascorbate peroxidase activity

Ascorbate peroxidase (APX, EC 1.11.1.11) was determined according to Nakano and Asada (1981) and with modified procedure of Hernàndez et al. (2004). One gram of fresh

leaves with removed veins was ground in 5 ml phosphate buffer (pH 7.0). The homogenate was centrifuged at 4,000 rpm for 10 min at 4°C. The assay medium consists of 3 ml containing 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.3 mM ascorbate, 0.06 mM H_2O_2 and 0.1 ml enzyme extract. The decrease in ascorbate concentration was followed by decline in absorbance at 290 nm and the activity was calculated using the extinction coefficient ($E = 2.8$ mM^{-1} cm^{-1} at 290 nm) for ascorbate.

Catalase activity

Catalase (CAT, EC 1.11.1.6) activity was measured according to Chandlee and Scandalios (1984), with some modifications. Catalase activity was measured by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm (extinction coefficient = 0.036 mM^{-1} cm^{-1}) of a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0), 20 mM H_2O_2 and enzyme extract. One CAT unit is defined as the amount of enzyme necessary to decompose 1 μmol min^{-1} H_2O_2 under the above-mentioned assay conditions.

Superoxide dismutase activity

Superoxide dismutase (SOD EC 1.15.1.1) activity was assayed according to Beauchamp and Fridovich (1971) with some modifications. The samples (0.5 g) were homogenized in 5 ml extraction buffer consisting of 50 mM phosphate, pH 7.8, 0.1% (w/v) ascorbate and 0.05% (w/v) β-mercaptoethanol. The assay mixture in 3 ml contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-Methionine, 75 μM NBT (nitroblue tetrazoline), 2 μM riboflavin and the enzyme extract. The photo-reduction of NBT was measured at 560 nm. One unit of SOD activity was defined as extract volume that caused 50% inhibition of the photo-reduction of NBT.

H_2O_2 concentration

H_2O_2 content was determined colorimetrically as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 0.5 g leaf tissue with 3 ml of phosphate buffer (50 mM pH 6.5). The homogenate was centrifuged at 6,000 rpm for 25 min. To determine H_2O_2 level, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium sulfate in 20% H_2SO_4. The mixture was then centrifuged at 6,000*g* for 15 min. The pellet was then dissolved in 5 ml of H_2SO_4 (2 M) and the intensity of the yellow color of the supernatant at 410 nm was measured. H_2O_2 level was calculated using the extinction coefficient ($E = 0.28$ μmol^{-1} cm^{-1}).

MDA concentration

Lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined as 2-thiobarbituric acid (TBA) reactive metabolites according to Zhang (1992). One gram of fresh leaves with removed veins was ground in 5% trichloroacetic acid (TCA) and then centrifuged at 3,000 rpm for 10 min. 2 ml of the supernatant was mixed with 2 ml of 0.03 M thiobarbituric acid (TBA) and incubated for 15 min at 94°C to develop the $(TBA)_2$-MDA adduct. The mixture was cooled using tap water and the absorbance was measured at wavelength 532 nm. Lipid peroxidation was expressed as nmol MDA $(g\ FW)^{-1}$ using an extinction coefficient ($E = 155$ $mM\ cm^{-1}$).

Flavonoids content

Content of flavonoids was measured in fresh leaves according to the method of Quettier-Deleu et al. (2000) with some modifications. Each extract of fresh leaves in methanol (0.5 ml) was mixed with 1 ml 5% sodium nitrite ($NaNO_2$) and after 5 min, 1.0 ml of 10% $AlCl_3{\cdot}6H_2O$ was added. The mixture was allowed to stand for 10 min and then 5 ml of 4% sodium hydroxide was directly added to the mixture. The absorbance was measured after 15 min at wavelength of 510 nm. Content of flavonoids was calculated on the basis of the calibration curves of Rutin trihydrate and was expressed as mg flavonoids per g fresh weight.

Total phenolics content

Total phenolics were determined using Folin–Ciocalteau reagents (Singleton and Rossi 1965). Gallic acid standard solution (2.0 mg/ml) was prepared by accurately weighing 0.01 g and dissolving 50 ml of distilled water. The solution was diluted to give with concentrations working standard solutions of 1.5, 1.0, 0.5, 0.2, and 0.1 mg/ml. 40 μl of extract (in 80% methanol) or gallic acid standard was mixed with 1.8 ml of Folin–Ciocalteu reagent (previously diluted tenfold with distilled water) and allowed to stand at room temperature for 5 min, and then 1.2 ml of $NaHCO_3$ (7.5% w/v) was added to the mixture. After standing 60 min at room temperature, absorbance was measured at 765 nm. Results are expressed as mg/g gallic acid equivalents (GAE).

DPPH free radical scavenging assay

The free radical-scavenging activity of leaf extract in methanol was measured using the method described by Shimada et al. (1992). Each extract (0.2–10 mg ml^{-1}) in methanol (2 ml) was mixed with 2 ml of freshly prepared

methanolic solution containing 80 ppm of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. The mixture was shaken vigorously and left to stand for 30 min in the dark. The absorbance was then measured at 517 nm. The percentage of DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging ability} = \left[1 - \left(A_i - A_j\right)/A_c\right] \times 100$$

where A_i is absorbance of extract + DPPH, A_j is absorbance of extract + methanol, and A_c is absorbance of DPPH + methanol. A lower absorbance indicates a higher scavenging effect.

Protein composition and protein patterns analyses

Protein contents

Soluble, insoluble and total protein contents of leaves were determined according to Lowery et al. (1951). A known weight of dry tissues was extracted in 10 ml distilled water for 2 h at 90°C for analysis of soluble protein. For total protein, 50 mg dry tissues were extracted in 10 ml NaOH (0.1 N) for 2 h at 90°C. The extracts were centrifuged and the supernatants were collected. 1 ml of extract was added to 5 ml of alkaline reagent (50 ml 2% Na_2CO_3 prepared in 0.1 N NaOH 1 ml 0.5% $CuSO_4{\cdot}5H_2O$ prepared in 1% sodium potassium tartarate), mixed thoroughly and then allowed to stand for 10 min. 0.5 ml of Folin reagent diluted 1:1 (v/v) was then added and mixed immediately. After 30 min, the extinction against appropriate blank was measured at 700 nm. Results were expressed as mg per g dry weight. Insoluble proteins were calculated as the difference between the amounts of total and water-soluble proteins. Bovine serum albumin was used for calibration curve.

Protein patterns

According to the procedure of Laemmli (1970), SDS-PAGE was done using 10% acrylamide gels. Extraction of protein samples (40 μg each) was performed by grinding 1 g fresh leaves in 0.5 ml Tris–HCl buffer in a precooled mortar. Samples were centrifuged at 14,000 rpm for 30 min at 4°C. Supernatants were mixed with an equal volume of loading buffer containing 0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol and bromophenol blue as tracking dye. The prepared mixture was then heated in water bath at 96°C for 3 min and loaded onto gel for 6 h at 10°C in run buffer containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Separated protein bands and polypeptides were visualized using 0.1% Commassie Brilliant R250 in destain solution. Finally the gels were destained in the destain solution containing 7% glacial acetic acid, 40% (v/v) methanol and 53% distilled water.

Statistical analyses

All data were subjected to ANOVA test and means were compared by two conventional methods of analysis. The LSD values for significant mean differences at levels $P < 0.05$ and $P < 0.01$ were separated. All statistical tests were carried out using SPSS software.

Results

Antioxidant enzyme activities

Bean leaves infected with BYMV showed induced POD and CAT activities. BYMV caused 29.6 and 56.0% increase in activities of POD and CAT, respectively, more than healthy control (Fig. 1). It was noticed that the 100 (μM SA + BYMV) level exhibited a significant increase of about 60% in POD activity while CAT activity inhibited by 15.2% less than control. Inhibitions of POD and CAT activities were detected with the increase of SA concentrations. In terms of increasing SA concentration, 10–100 μM (SA + BYMV) could inhibit POD activity from 106.5 to 53.9 Units g^{-1} FW and CAT activity from 110.3 to 72.02 Units g^{-1} FW (Fig. 1). Without virus inoculation, SA spraying could enhance peroxidase but inhibit catalase activity.

BYMV increased both the APX and SOD activities significantly (Fig. 2). This increase was 299% for APX and 37% for SOD compared with the control. With SA treatments, data concerning APX responses were variable while the activity of SOD increased markedly. Only the concentration of 100 μM SA with or without BYMV infection could affect APX by increasing its activity to be significantly higher than the value of the control. The increase in SOD activity occurred in a narrow range where the minimum and maximum increases reached 35.2 and 56.6%, respectively, regardless of the SA concentration (Fig. 2).

MDA and H_2O_2

BYMV induced higher amounts of MDA and H_2O_2 which reached 27 and 166.2%, respectively, more than the control (Figs. 3, 4). Bean leaves treated with SA showed insignificant changes of MDA contents in comparison to the control. Noticeably, the lowest MDA content was detected in 100 μM (SA + BYMV) treated leaves. H_2O_2 contents followed a gradual and significant increase with increasing SA concentration. The contents were 42.0 and 103.7% in 50 and 100 μM (SA + BYMV) treated leaves, respectively. Both 100 μM (SA + BYMV Mix) and 100 μM SA caused no changes in either MDA or H_2O_2 contents of faba bean leaves compared to the control.

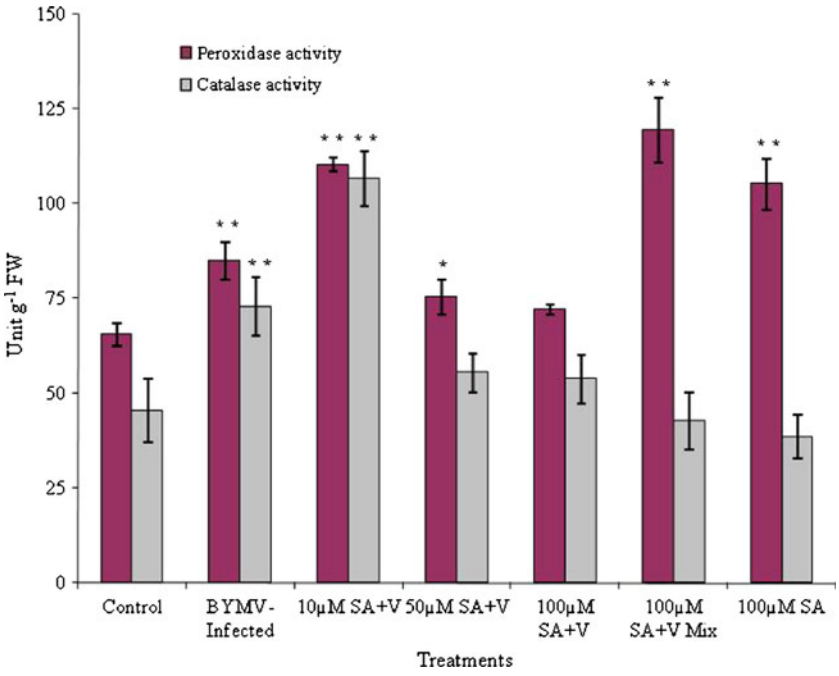

Fig. 1 Effect of BYMV infection and SA treatments on peroxidase and catalase activities (Unit g^{-1} FW) of faba bean (*Vicia faba* cv. Giza 461) leaves. Values are means (M) of four replicates ± standard deviation (SD). Statistical significance of differences compared to control: *significant at $P < 0.05$; **significant at $P < 0.01$

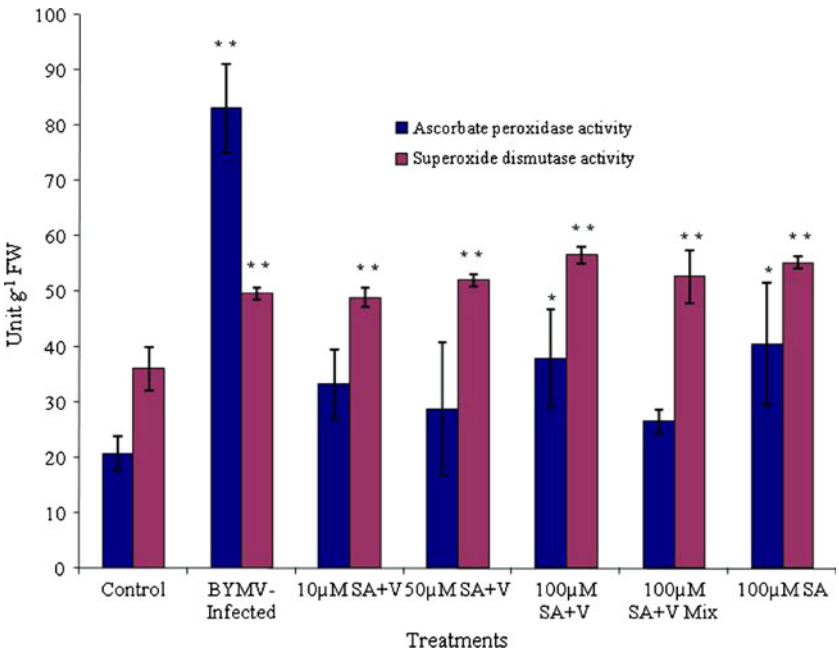

Fig. 2 Effect of BYMV infection and SA treatments on ascorbate peroxidase and superoxide dismutase activities (Unit g^{-1} FW) of faba bean (*Vicia faba* cv. Giza 461) leaves. Values are means (M) of four replicates ± standard deviation (SD). Statistical significance of differences compared to control: *significant at $P < 0.05$; **significant at $P < 0.01$

Total phenolics and flavonoids contents

With respect to the corresponding controls of bean leaves, the total phenolics reached 51.65% while flavonoids content reached 45.0% in infected bean leaves higher than those of the control (Figs. 5, 6). Bean leaves treated with SA showed highly significant increase of phenolics in all levels of treatment, especially, 100 µM (SA + BYMV) which accumulated phenolics by 47.9% over the control (Fig. 5). Similarly, flavonoid contents increased greatly with SA spray. From the results obtained (Fig. 6), SA appears to be the main factor in the induction of elevated amounts of flavonoids. The evidence for this phenomenon was the double-fold increase of flavonoids in 100 µM SA sprayed leaves and the gradual increase of contents with increasing SA concentration (Fig. 6).

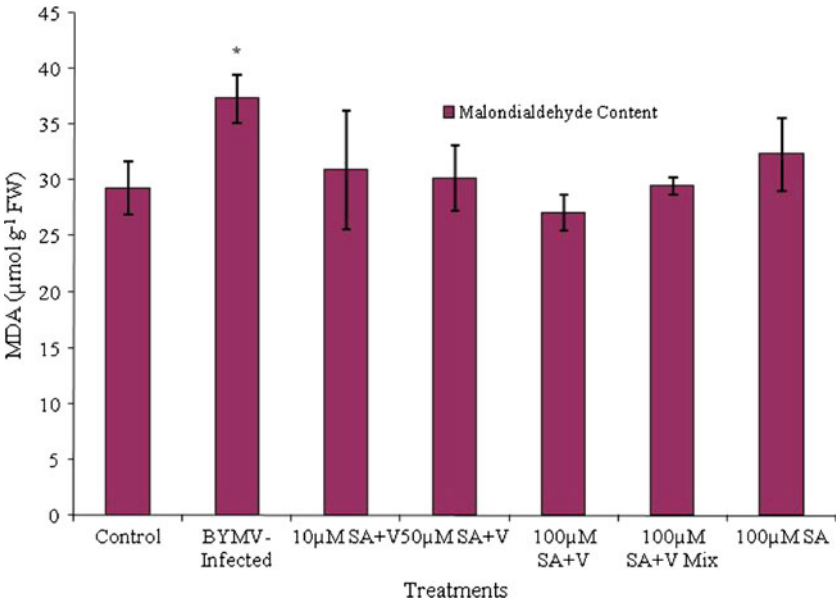

Fig. 3 Effect of BYMV infection and SA treatments on malondialdehyde content (µmol MDA g^{-1} FW) of faba bean (*Vicia faba* cv. Giza 461) leaves. Values are means (M) of four replicates ± standard deviation (SD). Statistical significance of differences compared to control: *significant at $P < 0.05$; **significant at $P < 0.01$

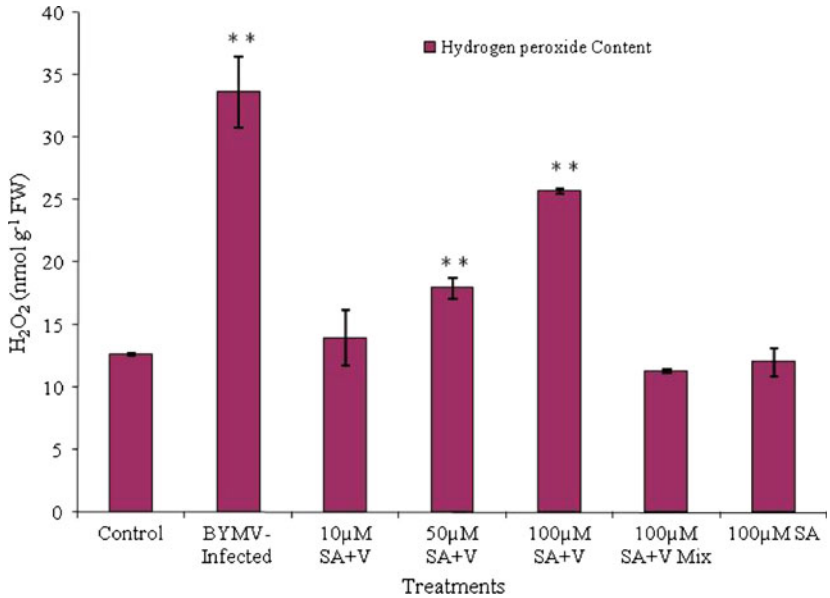

Fig. 4 Effect of BYMV infection and SA treatments on hydrogen peroxide content (nmol H_2O_2 g^{-1} FW) of faba bean (*Vicia faba* cv. Giza 461) leaves. Values are means (M) of four replicates ± standard deviation (SD). Statistical significance of differences compared to control: *significant at $P < 0.05$; **significant at $P < 0.01$

Total antioxidant activity (TAA) by DPPH scavenging assay

Figure 7 shows the effects of BYMV infection and SA treatments on TAA of faba bean leaves. BYMV affected bean leaves by increasing the antioxidant activity to 12% more than the control.

As compared to the control, SA addition caused pronounced increases in antioxidant activity of leaf extracts indicating an increase in the amounts of antioxidant compounds. However, BYMV inoculated leaves which exposed to 100 µM SA prior to inoculation showed the maximum increase in TAA. Moreover, 10 and 50 µM (SA + BYMV) also induced formation of antioxidants in bean leaves (Fig. 7).

Protein content

Contents of faba bean protein constituents (soluble, insoluble and total) of healthy and infected leaves as well as SA treated with or without virus inoculation are shown in Table 1. BYMV-infected bean leaves had protein contents higher than the control regardless of the chemical treatment. In most cases of SA treatments, an increase in protein amounts was noticed and the pattern of increase was in accordance with SA concentration.

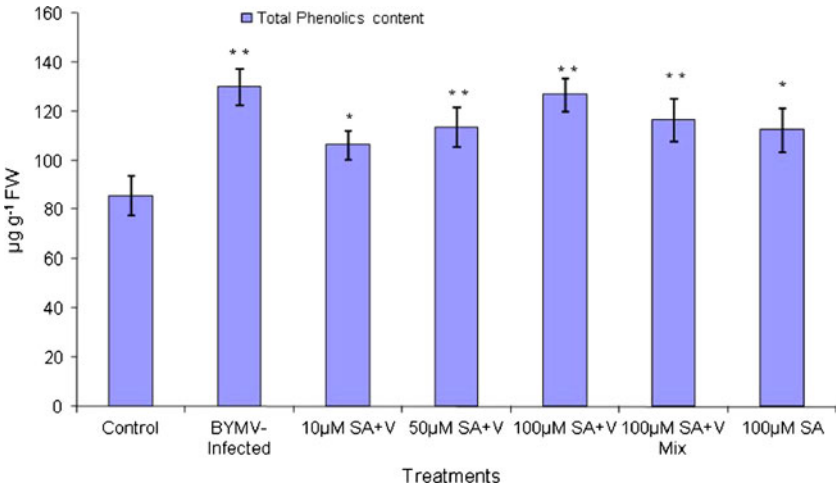

Fig. 5 Effect of BYMV infection and SA treatments on total phenolics content (µg g^{-1} FW) of faba bean (*Vicia faba* cv. Giza 461) leaves. Values are means (M) of four replicates ± standard deviation (SD). Statistical significance of differences compared to control: *significant at $P < 0.05$; **significant at $P < 0.01$

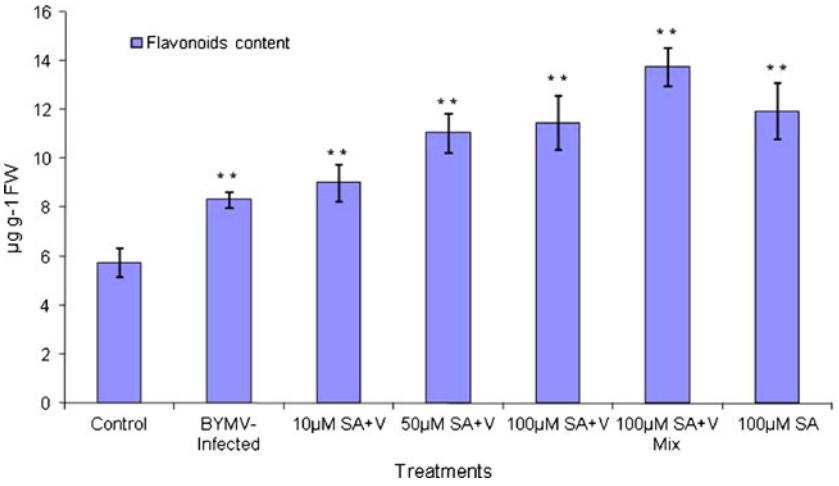

Fig. 6 Effect of BYMV infection and SA treatments on flavonoids content (µg g^{-1} FW) of faba bean (*Vicia faba* cv. Giza 461) leaves. Values are means (M) of four replicates ± standard deviation (SD). Statistical significance of differences compared to control: *significant at $P < 0.05$; **significant at $P < 0.01$

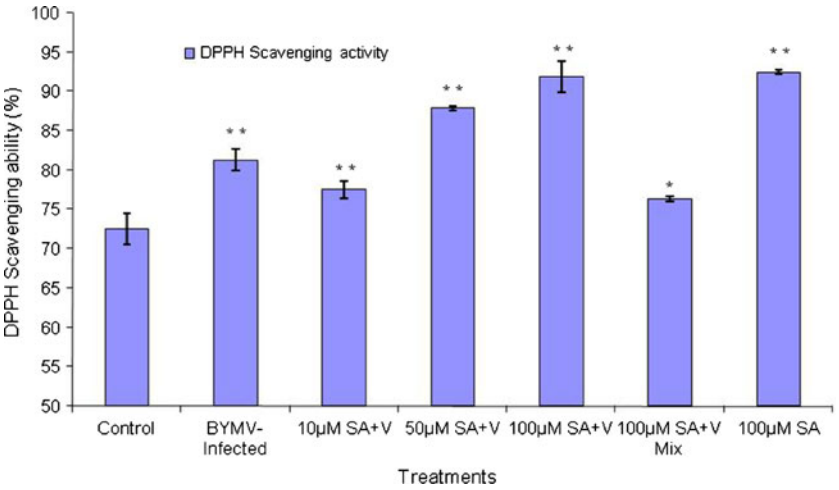

Fig. 7 Effect of BYMV infection and SA treatments on DPPH scavenging ability of leaf extract (%) of faba bean (*Vicia faba* cv. Giza 461) leaves. Values are means (M) of four replicates ± standard deviation (SD). Statistical significance of differences compared to control: *significant at $P < 0.05$; **significant at $P < 0.01$

For instance, 100 µM (SA + BYMV) caused an increase of about 51.5, 86.3 and 71.8%, for soluble, insoluble and total proteins, more than control (Table 1). Spraying SA without virus inoculation could accumulate soluble, insoluble and total proteins by 45.9, 31.2 and 37.3%, respectively. Induction of high amounts of proteins was thought to play a role in defence response against viruses.

Table 1 Effect of BYMV infections and SA treatments on protein contents (mg g^{-1} FW) of faba bean (*Vicia faba* cv. Giza 461) leaves

Treatments	Soluble proteins M ± SD (%)	Insoluble proteins M ± SD (%)	Total proteins M ± SD (%)
Control	90.73 ± 6.469 (100)	128.067 ± 9.322 (100)	218.80 ± 12.831 (100)
Infected (BYMV)	104.60 ± 1.0583 (115.283)*	167.400 ± 11.846 (130.713)*	272.00 ± 12.718 (124.314)**
10 μM SA + BYMV	109.20 ± 7.502 (120.353)**	205.200 ± 11.793 (160.229)**	314.40 ± 9.822 (143.693)**
50 μM SA + BYMV	126.67 ± 5.981 (139.603)**	209.333 ± 28.566 (163.457)**	336.00 ± 24.505 (153.565)**
100 μM SA + BYMV	137.47 ± 10.304 (151.506)**	238.533 ± 19.354 (186.257)**	376.00 ± 9.165 (171.846)**
100 μM SA + BYMV Mix	130.47 ± 7.572 (143.791)**	159.133 ± 13.400 (124.258)	289.60 ± 9.625 (132.358)**
100 μM SA	132.40 ± 2.800 (145.922)**	168.000 ± 40.341 (131.182*	300.40 ± 43.105 (137.294**

Values are means (M) of four replicates ± standard deviation (SD)

Statistical significance of differences compared to control: *significant *at* $P < 0.05$; **significant at $P < 0.01$

Protein patterns

Many alterations in protein patterns were observed in faba bean leaves in response to BYMV infection and SA treatments (Fig. 8). Due to BYMV infection and level of 10 μM (SA + BYMV), the protein profiles showed three new bands of weights 102, 90 and 36 kDa in comparison to the control. Generally, the intensity of protein bands of infected and 10 μM (SA + BYMV) lanes was less than that of the control (Fig. 8). In case of infected and 10 μM SA + BYMV), the polypeptide of 84 kDa showed lower intensity than the control; however, it was accumulated in other treatments. Besides the infected and 10 μM (SA + BYMV) cases, the profiles of protein pattern displayed a noticeable disappearance of two polypeptides of about 263 and 243 kDa (Fig. 8). Except for the 10 μM (SA + BYMV) level, the other SA treatments showed synthesis of new polypeptide of 68 kDa. A 36 kDa polypeptide which was not detected in the control, however, was observed in all treatments. The polypeptides of 147, 96, 84, 72 and 54 kDa were highly accumulated in treatments of 50, 100 μM (SA + BYMV), 100 μM (SA + BYMV) Mix and 100 μM SA. The polypeptide 54 kDa was more or less accumulated depending on the treatment level. A gradual decrease in band intensity of 35 kDa was noticed with increasing SA treatments which totally disappeared in treatment levels of 100 μM (SA + BYMV) and 100 μM SA (Fig. 8).

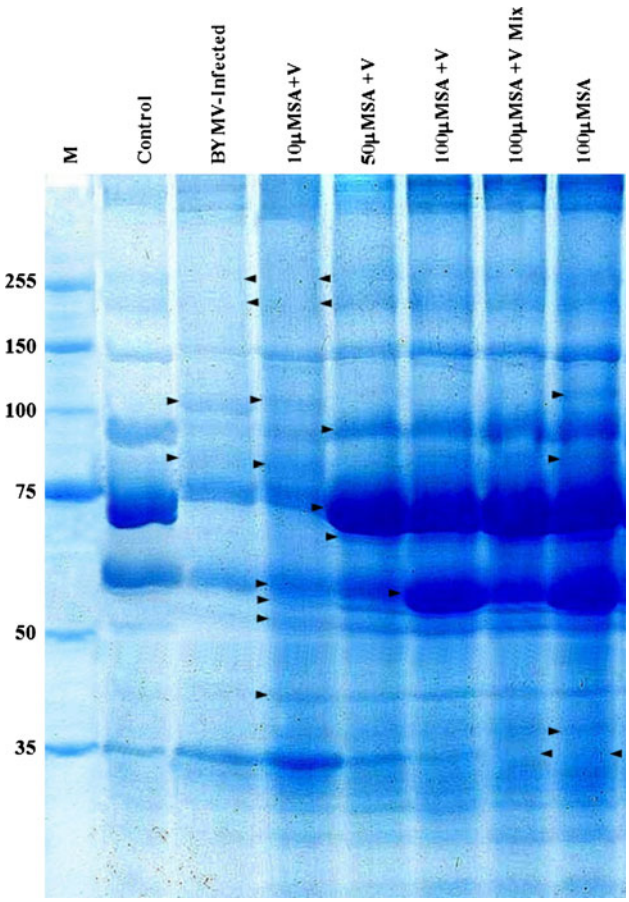

Fig. 8 SDS-PAGE profile showing the changes in protein patterns of bean leaves (*Vicia faba* cv. Giza 461) infected with BYMV and treated with 10, 50, 100 μM (SA + BYMV), 100 μM (SA + BYMV Mix) and 100 μM SA. *Arrow heads* point to the polypeptides newly appearing, disappearing modified under BYMV, SA or (SA + BYMV) treatments. The newly created proteins are considered as pathogenesis-related proteins. *Lane M* is the profile of standard proteins marker: 255, 150, 100, 75, 50, 35 and 25 kDa. Polypeptide bands were visualized using Coomassie brilliant blue stain

Discussion

The production of AOS in plant cells in response to virus infection is well documented (Riedle-Bauer 2000; Hernàndez et al. 2004; De Gara et al. 2003). AOS metabolism induces many changes in antioxidant enzyme

systems which are still under discussion. Many reports describe contradictory results with both induction and inhibition of antioxidant enzyme activities involved in free radical scavenging (Baker and Orlandi 1995; Clarke et al. 2002; Hernàndez et al. 2001).

The present results showed that the activities of POD, APX, CAT and SOD enzymes increased significantly with BYMV infection. The most obvious increase was caused by BYMV infection detected with APX activity where the increase reached 299% of the control. The results concerning POD and APX activities are in agreement with those reported by Riedle-Bauer (2000), who found a highly significant increase in POD and APX in both *Cucumis sativus* and *C. pepo* infected by ZYMV. Moreover, Milavec et al. (2001) reported an increase in POD activity of potato leaves infected with potato virus Y. To confirm, peroxidase shows increase in its activity in *Cucurbita pepo* plants infected with *Cucumber mosaic virus* (CMV) (Técsi et al. 1996) and *Zucchini yellow mosaic virus* (ZYMV) (Radwan et al. 2006). Moreover, the changes in these antioxidant enzymes are known to be directly involved in the activation of plant defense responses (Hernàndez et al. 2004; De Gara et al. 2003; Hernàndez et al. 2001).

Furthermore, increasing SA dose could inhibit POD, CAT activities but induced SOD activity. The activities seemed to be SA concentration dependant. Although there was a gradual decrease in POD with the increase of SA concentration, the values are still extremely higher than controls. Peroxidase is considered the first to show changes in its activity under stress (Milavec et al. 2001). POD is known to catalyze the final polymerization step of lignin synthesis and is directly associated with the increased ability of systemically protected tissues to lignify (Chittoor et al. 1999). Hence, suppression of POD, APX and CAT activities and induction of SOD reflect the role of SA in defense responses against virus infection.

Interestingly, an induction of H_2O_2 was recorded in this experiment under virus infection and SA treatments. In comparison to the control, BYMV-infected leaves showed accumulated H_2O_2 content. SA might be a reason to induce more H_2O_2 in leaves regardless of the virus infection. It is known that SA acts as electron donor for peroxidase isoforms, causes inhibition of the enzyme and leads to the production of SA free radicals. Inhibition of peroxidase leads to enhanced H_2O_2 accumulation causing an alteration in the cellular redox state.

Hence, application of SA induces at least one of the cellular protective mechanisms through potentiality of an oxidative burst. The mode of SA was proposed to increase H_2O_2 level by inhibiting CAT activity during plant–pathogen interactions. It was documented that the pretreatment with SA alone specifically inhibited CAT activity and increased H_2O_2 level in rice roots. H_2O_2 accumulation affected by SA has also been observed in other plant species (Chen et al. 1995, 1993). During pathogen attack in plants, AOS, including H_2O_2 accumulation, can act with at least one of three mechanisms against pathogens.

- First, H_2O_2 can act directly by killing the pathogen.
- Second, H_2O_2 can react with transition metals leading to enhanced generation of reactive hydroxyl radicals, the devastating effect on bio-molecules of which is well known.
- Third, H_2O_2 also hinders penetration of plants by micro-organisms. It contributes to cell wall stiffening by facilitating peroxidase reactions catalyzing intra- and inter-molecular cross-links between structural components of cell walls and lignin polymerization.

As a consequence, an increase in mechanical barriers slows down pathogen penetration allowing plant cells to arrange defenses which require more time to be activated. In addition, H_2O_2 can diffuse easily through biological membranes, so it acts as intercellular signal which is able to activate defense response against pathogen attack (De Gara et al. 2003).

Nowadays, development of morphological and epidemiological symptoms due to virus infection is associated with changes in antioxidant status and protein composition within leaves. AOS have been suggested to be involved in plant defence responses. Production of AOS can reinforce plant cell walls through cross linking reactions of lignin and protein. AOS are toxic agents against either the host plant cells, with development of hypersensitive response and systemic acquired resistance (SAR) or against the pathogens, killing them or stopping their growth and development. Moreover, AOS are considered as second messengers in signaling routes leading to the activation of plant defence related genes. AOS production has been established in several plant tissues to be associated with the expression of SAR in plant tissues and cell suspension culture systems (Ádám et al. 1989; Alvarez et al. 1998; Devlin and Gustine 1992; Doke 1983; Doke and Ohashi 1988; Thordal-Christensen et al. 1997; Apostol et al. 1989; Legendre et al. 1993; Levine et al. 1994; Vera-Estrella et al. 1992). In plant cells, AOS can directly cause strengthening of host cell walls via cross-linking of glycoproteins (Bradley et al. 1992; Lamb and Dixon 1997), or lipid peroxidation and membrane damage (Lamb and Dixon 1997; Montillet et al. 2005). AOS level is not only affected by antioxidant enzyme activities but also by the presence or absence of antioxidant metabolites such as phenolic compounds and flavonoids.

Phenolic compounds and flavonoids have strong free radicals scavenging capacity (Huang et al. 2006). In this work, an increase in the level of phenolics and flavonoids with either virus infection or SA treatments was observed.

To support, it is reported that phenolic compounds are considered as free radical scavenging molecules, which are rich in their antioxidant activity (Velioglu et al. 1998; Zheng and Wang 2001; Cai et al. 2003).

The antioxidant properties of phenolics are mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides and have health functional properties that may protect from various diseases (Heinonen et al. 1998; Rice-Evans and Miller 1998). From the present results, flavonoids were accumulated to be twofold higher than control in 100 μM SA level. Flavonoids mainly act as antioxidants, to prevent viral binding and penetration into cells, and to trigger the host cell self-defense mechanisms (Friedman 2007). This supports a role of SA in the induction of antioxidants and enhanced resistance. To confirm the accumulation of antioxidant metabolites in this experiment, a scavenging assay was performed for each treatment and the results revealed an increase in the antioxidant activity of leaf extract in response to SA spraying which points to SA as an agent that accumulates amounts of antioxidants.

Protein synthesis is essential for normal cell differentiation and growth. A variety of environmental stressors have been reported to influence the synthesis of plant proteins (William 1989). Plants responded to pathogen attack by formation of new families of proteins called pathogenesis-related proteins or PR proteins (van Loon 1985). In this work, protein contents as well as protein profile by means of SDS-PAGE were analyzed. Generally, induced amounts of soluble, insoluble and total proteins were detected in response to BYMV infection. Our results showed BYMV infection leads to increase of about 15.28, 30.7 and 24.3% for soluble, insoluble and total proteins, respectively. Accumulation of proteins in virus-infected plants was reported previously by Fraser (1982) as pathogenesis-related protein. As stated in the introduction, plants infected with the strong isolate of PVY had higher total content of PR proteins than healthy plants and plants infected with weak isolates and the greater the damage to the plant caused by virus infection, the more PR proteins are accumulated (Sapotsky et al. 2005).

Applying certain doses of SA to faba bean leaves caused prominent accumulation of total proteins. It is well known that establishment of SAR in plants is found to be associated with induced amounts of new proteins (Hoegen et al. 2002). As SA acts as a regulator promoting SAR inside the plant cell, it is suggested that protein accumulation occurs due to involvement of SA in protein synthesis mechanisms. In other words, SA plays a role in induction of defence mechanisms through systemic enhancement of PRPs. To support, Katoch (2007), found that treatments of pea leaves with SA results in pronounced increase in total protein content and formation of new proteins. Recently, SA application induces accumulation of PR proteins including SA-binding protein which plays a role in defence responses (Loake and Grant 2007). The PR proteins display several additional functions, including a role in the developmental processes and enzymatic activities in secondary metabolism (Liu and Ekramoddoullah 2006). Furthermore, the results of protein contents seem to be in parallel with those of protein patterns investigations by SDS-PAGE. Some new protein bands and a high increase in the density of other protein polypeptides were detected. BYMV-infected leaves showed newly synthesized polypeptides which are believed to be PRPs. Moreover, profile expressing SA treatments with faba bean showed a higher density of some protein bands. This indicates a role of exogenous SA in the induction of more defense proteins. Formation of new proteins and protein accumulation is considered a way and an indicator of resistance towards virus infection. It is reported that exogenous application of SA induces both resistance to TMV and the accumulation of PR proteins in tobacco plants (White 1979). Recently, it was found that, SA resulted in the appearance of a new protein band of 96.7 kDa in pea plant (Katoch 2007). In this experiment, SA treatments induce the formation of new protein bands of 36 and 68 kDa in faba bean leaves. This indicates that SA plays an important role in the induction of resistance. This role may occur through accumulation of certain proteins and/or formation of new polypeptides which are so-called PRPs.

In conclusion, a modified antioxidant status was noticed in faba bean leaves due to BYMV infection and SA treatments. The modifications include changes in the activities of some antioxidant enzymes, accumulation of phenolic compounds, flavonoids and antioxidant metabolites. Protein profiles were qualitatively and quantitatively altered in response to virus infection and SA treatments in bean leaves.

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