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Alleviation of oxidative stress induced by spider mite invasion through application of elicitors in bean plants

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Abstract

Spider mite invasion induces oxidative stress on bean plants and increased soluble sugars, phenole, proline and peroxidase activity, but decreased catalase activity and ascorbic acid and carotenoid concentration. Application of elicitors significantly enhanced spider mite tolerance by decreasing hydrogen peroxide, malondialdehyde, electrolyte leakage, and increasing peroxidase activity and antioxidant compounds reported previously, leading to increasing ion percentage in plant shoot. This finding suggests that elicitors might be activating antioxygenic enzymes and elevating antioxidants there by controlling free radical generation, hence preventing membrane peroxidation and denaturation of biomolecules resulting into improving plant growth.

Keywords: *Tetranychus urticae*, *Phaseolus vulgaris*, salicylate, methyl jasmonate, antioxidants.

Introduction

With a projected increase in world population to 10 billion over the next four decades, an immediate priority for agriculture is to achieve maximum production of food and other products in a manner that is environmentally sustainable and cost-effective. Losses due to insect herbivores, estimated at 10–20% for major crops, are a significant factor in limiting food production. The Two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae) is considered a major pest decreasing plant growth and yield on different agricultural crops including bean plants (Farouk & Osman 2009).

In response to pest invasion, plants mount a broad range of defence responses, including the generation of reactive oxygen species (ROS; Mur et al 2005) such as superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, which may initiate destructive oxidative processes such as chlorophyll bleaching, lipid peroxidation, protein oxidation, and damage to nucleic acids, leading finally to cell death (Lu & Finkel 2008, Nasir Kham *et al.* 2010). ROS also have been associated with oxidative changes in plants associated with oxidative damage in the midgut of insects feeding on previously wounded plants (Orozco-Cárdenas & Ryan 1999) or in *Spodoptera littoralis* feeding on lima bean (Maffei *et al.* 2006). Wound-induced ROS accumulation, in particular hydrogen peroxide (H_2O_2), is observed both locally and systemically in leaves of several plant species (Maffei *et al.* 2006). Being the most stable form of ROS, H_2O_2 can move into the cell membrane and initiate oxidative damage in leaf cells, resulting in disruption of metabolic function and loss of cellular integrity. H_2O_2 also changes the redox status of the surrounding cells and gives an antioxidative response by acting as a signal of oxidative stress (Hung *et al.* 2005). Available information suggests that H_2O_2 directly regulates the expression of numerous genes, some of which are involved in plant defence and the hypersensitive response, antioxidants, cell rescue/defence proteins and signaling proteins such as kinase, phosphatase and transcription factors (Henry 2008). In general, a plant's direct defence response upon herbivory is characterized by the activation of signalling cascades, which lead to the formation of specific products and physiological changes that interfere with the performance of the herbivore.

To defend themselves against insect attack, plants have evolved elaborate constitutive and inducible defence mechanisms. Constitutive defences are permanent structural or chemical

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compounds that occur regardless of the presence of damage and are determined by genetics, environment and former individual life history (Franceschi *et al.* 2005). For example, plants synthesize different types of antioxidant compounds and activate key antioxidant enzymes such as catalase (CAT; EC 1.11.1.6) and peroxidase (POD; EC 1.11.1.7) (Ni *et al.* 2001, Zhu *et al.*, 2004); phloem-feeding alfalfa hoppers (*Spissistilus festinus* L.) increase the activities of several oxidative enzymes and lipid peroxidation of soybean plants (Felton *et al.* 1994). It has also been reported that herbivory of plants generally stimulated an accumulation of proline and a decrease in total carbohydrate content, while there was no significant effect on the phenolics (Ni *et al.* 2001). Recent studies have also shown that *Nilaparvata lugens* infestation reduces the nutrient uptake of rice plants, especially phosphorous and potassium (Wu *et al.* 2004).

Traditionally, TSSM has been controlled using synthetic chemical acaricides with a level of residuality and permanence that constitute a barrier to the commercialization of agricultural products and cause detrimental effects to environment and human health. If used properly, they can control high populations of TSSM, but its phytophagous nature, high reproductive potential and short life cycle facilitate rapid resistance development to many acaricides often after only a few applications (Anazawa *et al.* 2003). Currently, great efforts are directed towards reductions in the use of traditional pesticides and increases in the use of Integrated Pest Management (IPM) techniques. One alternative pest control strategy involves the induction of a plant's own resistance mechanisms against pests by the application of elicitors. In recent decades, several plant chemical elicitors such as salicylic acid (SA) and jasmonic acid (JA) and its methyl ester (MeJA) have been used to induce defensive responses equivalent to insect or pathogen attacks (e.g. Hudgins & Franceschi 2004, Erbilgin *et al.* 2006). All these substances are endogenous plant phytohormones known to be involved in triggering induced defence responses after insect attack (Halitschke & Baldwin 2004).

JA and MeJA are one of the simplest of non-traditional plant hormones with diverse roles and functions, including a potential role in plant defence as part of a complex signalling pathway (Cheong & Choi 2003). In recent years, MeJA has been found not only to regulate a variety of plant-developmental responses, but to be induced by pathogen attack or wounds caused by herbivory that often lead to the generation of reactive oxygen species (Devoto & Turner 2003) and induced resistance to herbivores (Omer *et al.* 2001). JA is known to be produced by a plant after caterpillar damage, resulting in increased production of compounds involved in resistance (Thaler *et al.* 1996), including hydrogen peroxide, superoxide anions and hydroxyl free radicals (Devoto & Turner 2003). These observations suggest that MeJA could be linked to oxidative stress. The different effects of MeJA on protective-enzyme activities could be associated with H_2O_2 metabolism (Ali *et al.* 2006, Parra-Lobato *et al.* 2009).

Salicylic acid (SA), a plant phenolic and considered to be a hormone-like endogenous regulator, has been proposed as an endogenous signal associated with regulating oxidant levels in response to biotic stress. Recent studies have shown that SA plays an important role in provoking plant resistance to various biotic and abiotic stresses (Drazic & Mihailovic 2005, Farouk *et al.* 2008, Farouk & Osman 2009). Since the mode of the SA-signalling pathway is associated with increased H_2O_2 levels (Chen *et al.* 1993), it might be expected that SA pretreatment would accentuate the oxidative stress caused by TSSM invasion. Pretreatment with SA protects plants from oxidative damage, and is often associated with an increase in antioxidant enzymes (Daneshmand *et al.* 2009). Mahdavian *et al.* (2007) on pepper showed that exogenous application of SA caused significant increases in polyphenol oxidase and peroxidae enzyme activities, but decreased catalase, ascorbate peroxidase and glutathione reductase activities.

The maintenance of membrane integrity is important for enhanced stress tolerance. This is why the role of SA or MeJA on membrane leakage, lipid peroxidation, H_2O_2 production and mineral uptake of plants under TSSM invasion is of great importance. Therefore, the induction of plant resistance against herbivores is considered to be a possible potent method in reducing

and controlling red mite infestion. To our knowledge, however, little is known about the dynamics of the activities of antioxidants under TSSM infestion. The present study was an attempt to carry out an investigation into the effect of SA and MeJA on bean plants under TSSM infestation, with the aim of characterizing variation in antioxidant ability of different concentrations, investigating the possible mechanisms responsible for TSSM tolerance in treated plants treated with SA or MeJA, and elucidating the possible mechanisms that might be involved in the SA- or MeJA-promoted antioxidant responses to TSSM infestation.

Materials & Methods

Two field experiments were conducted in the Plant Protection greenhouse of the Faculty of Agriculture in Mansoura University in two successive seasons (2006 and 2007) to study the impact of MeJA and SA on TSSM infestation in common bean (*Phaseolus vulgaris*) plants, and on inducing plant resistance to TSSM. Seeds of bean cv. Bronco were sown on March 12 and 9 in the two consecutive seasons respectively. Seeds were sown in hills 10 cm apart on one side of ridges 3 m long and 60 cm width. All agricultural practices were carried out according to the recommendation of Ministry of Agriculture, Egypt.

Plants were divided into four treatment groups within a complete randomized-block design consisting of four replicates per treatment. Treatment one consisted of infestation with 50 adult females of *T. urticae* per plant dispersed over the two trifoliate leaves of 15-day-old plants. The second group was infested the same way but then sprayed with one of the following solutions; distilled water, 50 or 100 mg/l SA, and $5x10^{-5}$ or 10^{-5} M MeJA, two weeks after the infestation (35, 40 and 45 days after sowing). Spraying was conducted by first spraying the plants with elicitor solution until dripping after adding 1% tween 20 as a wetting agent. Spraying was repeated i.e. the first time 21, the second 26 and the third 31 days post-infestation. The third group was sprayed the same way as the second treatment group and was then infested with mites after the third spray. The fourth group were uninfested control plants.

All plants were covered with mite-proof mesh bag. At full blooming stage (70 days after sowing) a random sample of five plants from each experimental plot was taken for determining hydrogen peroxide, lipid peroxidation, membrane permeability, ion content, antioxidant enzymes and compounds (catalase, peroxidase, ascorbic acid, proline, soluble carbohydrates, carotenoids, total phenolic compounds) of the bean shoot. Hydrogen peroxide content was determined according to the method of Velikova *et al.* (2000). Fresh plant materials were homogenized with 0.1% (w/v) trichloracetic acid in an ice bath. The homogenate was centrifuged at 10,000 g for 10 min and an aliquot from the supernatant mixed with 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI. The absorbance of the mixture was read at 390 nm. The content of H_2O_2 was given based on a standard curve.

Lipid peroxidation was estimated as thiobarbituric-acid-reactive substances. the malondialdehyde content was determined and calculated as µmoles/g of fresh weight by the method of Shao et al (2005). Leaf samples were homogenized in 5% (w/v) trichloracetic acid in an ice bath, and centrifuged at 10,000 g for 10 min at 4 ^{0}C. An equal volume of thiobarbituric acid in 20% trichloroacetic acid solution was added and the sample incubated at 95 °C for 30 min. The reaction was stopped by placing the reaction tubes in an ice bath. The samples were then centrifuged at 10,000 g for 30 min. The supernatant was removed, absorption read at 532 nm, and the amount of nonspecific absorption at 600 nm read and subtracted from this value. The amount of malondialdehyde present was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

Electrolyte leakage percentage measurement was used to assess membrane permeability according to Goncalves *et al.* (2007), using an Electrical Conductivity Meter (Hanna, UK). Leaf samples were placed in vials containing distilled water and incubated at

room temperature for 24 h. Electrical conductivity of the resulting solution (EC_1) was recorded after incubation. Samples were then placed in a boiling water bath for 30 min, cooled to room temperature, and the second reading (EC_2) determined. The electrolyte leakage percentage was calculated as EC_1/EC_2 and expressed as a percentage.

Catalase (CAT) (EC 1.11.1.6) activity was assayed by measuring the rate of disappearance of H_2O_2 using the method of Chance & Maehly (1955). The reaction mixture contained 100 mM phosphate buffer (pH 7.4), 30 mM H_2O_2 and enzyme extract. The reaction was stopped after 1 min by adding 10 ml of 2% H_2SO_4. The zero-time activity was carried out by adding 10 ml of 2% H_2SO_4 before the enzyme extract was added to the reaction. The acidified reaction mixtures were titrated with 0.01 N $KMnO_4$ until a faint pink color persisted for one minute.

Peroxidase (EC 1.11.1.7) activity was assayed by the method of Kumar & Khan (1982). The reaction mixture used for estimating the peroxidase enzyme contained 0.1 M phosphate buffer (pH 6.8), 0.01 M pyrogallol, 0.005 M H_2O_2 and the enzyme extract. The solution was incubated for 5 min at 25 ^{0}C after which the reaction was terminated by adding 2.5 N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a reagent blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at the zero time.

Ascorbic acid was extracted from plant material and titrated using 2.6- dichlorophenol indophenole, as described by Sadasivam & Manickam (1996). Total phenolic compounds were determined according to the method of Singleton & Rossi (1965) using Folin-Ciocalteau reagent. Proline was determined by the method described by Bates *et al.* (1973). Total soluble carbohydrates were extracted and estimated using the dinitrosalycylic acid method as described by Sadasivam & Manickam (1996). For the analysis of certain elements, one gram of the ground dry leaves was digested with acid mixture (perchloric and sulphuric acids as described by Chapman & Pratt (1978)). Total nitrogen content was determined using micro-Kjeldahl methods; potassium using a flame photometer (Kalra 1998), and phosphorous using ammonium molybdate and ascorbic acid (Cooper 1977).

The data were evaluated using one-way ANOVA to compare the effectiveness of elicitors in inducing plant resistance to TSSM. Means were then compared using Duncan's multiple-range test (at P=0.05) using COSTAT software.

Results

One of the expected consequences of TSSM-infestation-induced cellular build-up of reactive oxygen species is an increase in lipid peroxidation. The assay of cellular accumulation of lipid peroxidation products, in the form of thiobarbituric-acid-reactive substances, can provide a comparative indication of such activity. In the present study, the content of thiobarbituric-acid-reactive substances and membrane permeability were utilized as biomarkers for lipid peroxidation. Infestation with TSSM induced the accumulation of thiobarbituric-acid-reactive substances in bean shoots, followed by an increase in membrane permeability due to the hyper-accumulation of hydrogen peroxide in the two growing seasons (Table 1). The application of either SA or MeJA, and in particular 100 mg/l SA, before or after infestation significantly decreased the formation of thiobarbituric-acid-reactive substances and improved membrane permeability compared with control or untreated infested plants. The application of any elicitor significantly increased hydrogen peroxide content over control plants, but decreased it relative to untreated infested plants.

Table 1: Hydrogen peroxide and lipid peroxidation concentration as well as membrane permeability percentage of 70-day-old bean plants infested with Two-spotted Spider Mite (TSSM) and treated with salicylic acid (SA, at high [100 mg/l] or low [50 mg/l] levels) or the methyl ester of jasmonic acid (MeJA, at high [5 x 10^{-5} M] or low [10^{-5} M] levels).

Treatment		Hydrogen peroxide		Lipid peroxidation		Membrane permeability	
		1st season	2nd season	1st season	2nd season	1st season	2nd season
Control		15.4 ± 0.1^{h}	15.0 ± 0.3^{g}	9.2 ± 0.2^{b}	9.3 ± 0.2^{b}	81.1 ± 1.3^{d}	81.8 ± 0.8^{bcd}
TSSM		32.0 ± 0.5^{a}	31.7 ± 0.6^{a}	15.6 ± 0.5^{a}	16.8 ± 0.5^{a}	90.8 ± 0.7^{a}	90.9 ± 0.3^{a}
Infested and sprayed	high MeJA	21.0 ± 0.1^{bc}	21.7 ± 1.1^{b}	8.4 ± 0.1^{cd}	8.4 ± 0.1^{c}	86.3 ± 0.6^{bc}	86.2 ± 0.3^{ab}
	low MeJA	21.6 ± 0.2^{b}	21.3 ± 0.1^{bc}	8.7 ± 0.2^{bc}	8.6 ± 0.1^{c}	87.0 ± 0.1^{b}	86.2 ± 0.3^{ab}
	low SA	20.2 ± 0.2^{cd}	20.0 ± 0.1^{cd}	8.1 ± 0.03^{cd}	8.0 ± 0.1^{cd}	79.5 ± 0.7^{d}	79.5 ± 0.8^{bcd}
	high SA	19.0 ± 0.2^{e}	18.8 ± 0.2^{de}	7.3 ± 0.2^{e}	6.7 ± 0.2^{e}	77.1 ± 0.7^{e}	76.6 ± 0.4^{cde}
Sprayed then infested	high MeJA	18.2 ± 0.2^{f}	17.8 ± 0.1^{ef}	6.3 ± 0.1^{f}	6.0 ± 0.1^{f}	74.0 ± 0.4^{f}	73.9 ± 0.9^{de}
	low MeJA	19.6 ± 0.1^{de}	18.5 ± 0.9^{de}	7.8 ± 0.1^{de}	7.6 ± 0.1^{d}	84.5 ± 0.5^{c}	84.2 ± 0.6^{abc}
	low SA	17.4 ± 0.2^{g}	17.2 ± 0.3^{ef}	5.8 ± 0.2^{f}	5.4 ± 0.2^{f}	71.9 ± 1.1^{f}	68.8 ± 8.2^{e}
	high SA	16.0 ± 0.3^{h}	16.3 ± 0.2^{fg}	5.0 ± 0.1^{g}	4.3 ± 0.3^{g}	67.9 ± 1.0^{g}	68.6 ± 1.2^{e}

Values are given as mean ± SE of three replicates. Means in columns with different letters are significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

Data presented in Table 2 indicate that peroxidase activity significantly increased with either TSSM invasion alone, or the exogenous application of either SA or MeJA after or before infestation. Higher activities were obtained when plants were sprayed with either MeJA or SA before TSSM invasion, with the highest values with 100 mg/l SA before TSSM invasion in both growing seasons as compared with control or infested plants. Catalase activity decreased significantly due to TSSM infestation or SA application compared with control plants, but SA and in particular at 100 mg/l significantly increased activity over TSSM-infested plants. MeJA, and in particular when applied at a level of 10^{-5} M after TSSM infestion, significantly increased catalase activity over control or untreated infested plants.

Table 2: Catalase and peroxidase enzyme activities of 70-day-old bean plant shoots infested with TSSM and treated with SA or MeJA. Abbreviations as Table 1.

Treatment		Catalase		Peroxidase	
		1st season	2nd season	1st season	2nd season
Control		54.8 ± 0.7^{de}	54.5 ± 0.8^{d}	15.5 ± 0.2^{h}	15.4 ± 0.7^{h}
TSSM		34.1 ± 0.3^{i}	34.3 ± 0.6^{h}	22.2 ± 0.2^{g}	21.9 ± 0.3^{g}
Infested and sprayed	high MeJA	64.0 ± 0.7^{b}	62.0 ± 0.4^{b}	24.1 ± 0.2^{f}	23.5 ± 0.2^{f}
	low MeJA	69.8 ± 1.1^{a}	70.0 ± 1.2^{a}	23.1 ± 0.2^{g}	22.9 ± 0.2^{fg}
	low SA	43.5 ± 0.7^{h}	40.9 ± 0.8^{g}	26.3 ± 0.1^{de}	25.8 ± 0.2^{e}
	high SA	46.6 ± 0.7^{g}	46.5 ± 1.0^{f}	28.8 ± 0.5^{c}	28.6 ± 0.3^{c}
Sprayed then infested	high MeJA	57.0 ± 0.4^{d}	56.7 ± 0.6^{c}	27.1 ± 0.3^{d}	27.2 ± 0.2^{d}
	low MeJA	59.5 ± 0.6^{c}	58.5 ± 0.3^{c}	25.5 ± 0.2^{e}	25.1 ± 0.03^{e}
	low SA	51.0 ± 1.4^{f}	50.1 ± 0.9^{e}	31.5 ± 0.6^{b}	30.9 ± 0.7^{b}
	high SA	53.4 ± 0.4^{e}	52.1 ± 0.2^{e}	34.4 ± 0.3^{a}	34.3 ± 0.5^{a}

Values are given as mean± SE of three replicates. Means in columns with different letters are significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

Table 3: Ascorbic acid, soluble sugars, total soluble phenol, proline and total carotenoids of 70-day-old common bean plants infested with TSSM and treated with SA or MeJA in both growing seasons. Abbreviations as in Table 1.

Treatment		Ascorbic acid		Soluble carbohydrates		Total soluble phenol		Proline		Total carotenoids	
		1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control		15.7±0.4^{e}	15.9±0.2^{e}	29.2±2.6^{h}	27.3±2.0^{h}	11.3±0.1^{h}	11.4±0.4^{h}	11.6±0.5^{h}	15.0±0.3^{h}	0.53±0.01^{h}	0.52±0.01^{g}
TSSM		9.3±0.2^{f}	9.4±0.9^{f}	36.2±0.6^{g}	36.2±1.2^{g}	21.3±0.2^{g}	20.9±0.1^{g}	19.6±0.3^{g}	31.7±0.6^{g}	0.40±0.002^{i}	0.39±0.002^{h}
Infested and sprayed	high MeJA	18.1±0.6^{d}	18.1±0.6^{d}	48.0±1.0^{e}	46.5±0.8^{e}	23.9±0.6^{f}	23.4±0.4^{f}	22.0±0.2^{e}	21.7±1.1^{e}	0.71±0.005^{f}	0.70±0.01^{e}
	low MeJA	17.6±0.3^{d}	17.4±0.3^{de}	43.0±0.8^{f}	42.7±1.0^{f}	22.1±0.4^{fg}	22.7±0.3^{fg}	20.6±0.1^{f}	21.3±0.1^{f}	0.65±0.01^{g}	0.64±0.01^{f}
	low SA	19.1±0.4^{d}	19.1±0.4^{d}	50.9±0.5^{de}	50.1±0.3^{d}	27.9±0.6^{e}	27.3±0.3^{e}	23.3±0.1^{d}	20.0±0.1^{d}	0.74±0.01^{ef}	0.73±0.01^{de}
	high SA	22.6±0.1^{b}	22.2±0.5^{bc}	56.6±0.8^{c}	55.8±0.8^{c}	36.7±1.1^{c}	36.4±1.2^{c}	24.5±0.3^{c}	18.8±0.2^{c}	0.87±0.00^{c}	0.86±0.002^{b}
Sprayed then infested	high MeJA	20.8±0.3^{c}	20.8±0.6^{c}	60.7±0.6^{b}	59.9±0.6^{b}	34.6±0.2^{d}	32.9±1.5^{d}	25.2±0.2^{c}	17.8±0.1^{c}	0.83±0.01^{cd}	0.82±0.01^{bc}
	low MeJA	18.9±0.4^{d}	19.2±0.3^{d}	53.7±0.9^{cd}	52.9±0.7^{cd}	26.3±0.4^{e}	26.1±0.6^{e}	23.8±0.1^{d}	18.5±0.9^{d}	0.79±0.01^{de}	0.78±0.01^{cd}
	low SA	24.1±0.5^{b}	22.9±0.6^{b}	63.6±1.2^{b}	62.8±0.8^{b}	42.1±0.8^{b}	41.9±0.6^{b}	26.7±0.2^{b}	17.2±0.3^{b}	1.09±0.01^{a}	1.03±0.02^{a}
	high SA	27.7±1.3^{a}	26.1±0.7^{a}	67.3±0.8^{a}	66.9±0.6^{a}	46.5±1.1^{a}	46.1±0.2^{a}	27.9±0.2^{a}	16.3±0.2^{a}	1.03±0.05^{b}	0.99±0.04^{a}

Values are given as mean ± SE of three replicates. Means in columns with different letters are significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

The pattern of changes in antioxidant compounds is presented in Table 3. Plants infested with TSSM had the minimum concentrations of ascorbic acid and total carotenoids but significantly increased concentrations of soluble carbohydrates, total soluble phenol and proline concentration compared with control plants in both growing seasons. The application of both elicitors at both concentrations used, in particular 100 mg/l SA, increased significantly the concentrations of several antioxidant compounds, i.e. ascorbic acid, soluble carbohydrates, total soluble phenol, proline and carotenoids, compared with control or untreated infested plants. Higher antioxidant concentrations were obtained from applying SA at 100 mg/l before infestation (Table 3). The application of SA was more effective than MeJA in increasing antioxidant concentrations.

There were significant decreases in the percentage of nitrogen, phosphorous and potassium of TSSM-infested bean shoots as compared with healthy control plants (Table 4). The application of both elicitors before or after TSSM infestation significantly increased the percentage of nitrogen, phosphorous and potassium as compared with healthy or untreated infested plants in both growing saesons. Again SA was more effective than MeJA. The highest values were obtained from the application of 100 mg/l SA before TSSM infestation, with the second-highest value from 50 mg/l SA.

Table 4: Nitrogen, phosphorous and potassium percentages in 70-day-old bean plant shoots infested with TSSM and treated with SA or MeJA. Abbreviations as in Table 1.

Treatment		Nitrogen		Phosphorous		Potassium	
		1st season	2nd season	1st season	2nd season	1st season	2nd season
Control		3.08 ± 0.04^{f}	3.06 ± 0.06^{g}	0.36 ± 0.01^{h}	0.35 ± 0.02^{g}	0.86 ± 0.06^{g}	0.83 ± 0.06^{f}
TSSM		2.73 ± 0.11^{g}	2.73 ± 0.11^{h}	0.25 ± 0.04^{i}	0.24 ± 0.04^{h}	0.67 ± 0.02^{h}	0.64 ± 0.05^{g}
Infested and sprayed	high MeJA	3.38 ± 0.06^{e}	3.38 ± 0.08^{ef}	0.44 ± 0.002^{fg}	0.43 ± 0.01^{ef}	1.07 ± 0.01^{ef}	1.07 ± 0.02^{de}
	low MeJA	3.15 ± 0.04^{f}	3.15 ± 0.04^{fg}	0.40 ± 0.01^{gh}	0.40 ± 0.01^{fg}	1.00 ± 0.02^{f}	0.98 ± 0.02^{e}
	low SA	3.59 ± 0.09^{d}	3.50 ± 0.12^{e}	0.47 ± 0.01^{ef}	0.46 ± 0.01^{de}	1.14 ± 0.02^{e}	1.13 ± 0.02^{d}
	high SA	4.11 ± 0.06^{c}	4.08 ± 0.08^{c}	0.54 ± 0.01^{cd}	0.52 ± 0.005^{cd}	1.39 ± 0.02^{c}	1.37 ± 0.00^{c}
Sprayed then infested	high MeJA	4.29 ± 0.06^{bc}	4.25 ± 0.06^{bc}	0.57 ± 0.02^{c}	0.58 ± 0.01^{bc}	1.50 ± 0.01^{b}	1.49 ± 0.01^{b}
	low MeJA	3.76 ± 0.08^{d}	3.76 ± 0.06^{d}	0.49 ± 0.01^{de}	0.50 ± 0.01^{d}	1.27 ± 0.03^{d}	1.28 ± 0.03^{c}
	low SA	4.48 ± 0.04^{b}	4.43 ± 0.08^{b}	0.63 ± 0.004^{b}	0.62 ± 0.004^{b}	1.55 ± 0.01^{b}	1.55 ± 0.01^{b}
	high SA	4.74 ± 0.05^{a}	4.76 ± 0.07^{a}	0.69 ± 0.02^{a}	0.71 ± 0.04^{a}	1.69 ± 0.02^{a}	1.69 ± 0.03^{a}

Values are given as mean ± SE of three replicates. Means in columns with different letters are significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

Discussion

In response to attack, plants mount a broad range of defence responses, including the generation of ROS (Leitner *et al.* 2005), comprising radicals and other non-radicals: among these, much attention has been focused on hydrogen peroxide (Grant & Loake 2000), which is extremely reactive and can oxidize biological molecules, causing oxidative stress (Van Breusegem *et al.* 2001). Production and removal of ROS are strictly controlled under normal biotic stresses, but when higher plants are subjected to TSSM infestation, the equilibrium between production and scavenging of ROS is broken, resulting in oxidative damage to proteins, DNA and lipids (Mittler 2002). Lipid peroxidation measured as the amount of thiobarbituric-acid-reactive substances or malondialdehyde is produced when polyunsaturated fatty acids in the membrane undergo oxidation by the accumulation of free oxygen radicals. Bi & Felton (1995) have proposed that herbivore attacks can cause localized oxidative responses in soybean leaves and have identified some potential functions of ROS that might affect plant-herbivores interaction. Increases in malondialdehyde and H_2O_2 levels, and the rapid degradation of chlorophyll, were observed in bean plants infested with TSSM (Farouk & Osman, 2009, Table 1), suggesting that the observed plant-growth inhibition and yield reduction must be directly or indirectly attributable to TSSM-infestation-induced oxidative damage.

The reactive nature of ROS makes them potentially harmful to cellular components. Fortunately, plants have the capacity to cope by eliminating them with an efficient ROS-scavenging system. Jiang *et al.* (1994) demonstrated antioxidant enzymes such as superoxidase dismutase and catalase are directly involved in ROS scavenging and plant protection against ROS damage. After TSSM infection the plant can efficiently scavenge the ROS by this antioxidant defence system without any detectable effects on plant growth and yield. However, during some time periods, the scavenging system may become saturated by the increased rate of radical production, and ultimately the excessive levels of ROS result in severe cellular damage or trigger a genetically controlled cell-death program or decreased plant growth and yield (Van Breusegem *et al.* 2001, Farouk & Osman 2009). There are reports in this and other investigations that TSSM and other pest infestations increase the content of H_2O_2 and peroxidation of the lipid membrane, thus disrupting its permeability, or induce oxidative stress in plant tissues followed by increasing electrolyte leakage and decreasing ion percentage due to the inhibition of H_2O_2-scavenging enzymes, in particular catalase (Table 2). Decreased catalase activity in infested plants might promote H_2O_2 accumulation, which could result in a Haber–Weiss reaction to form hydroxyl radicals. Since OH radicals are known to damage biological membranes and react with most compounds present in biological systems, they might hasten lipid peroxidation and membrane damage in infested plants. However, the levels of ROS and the extent of oxidative damage depend largely upon the whole antioxidant defence system and the co-operation or coordination among ROS-scavenging enzymes (Liang *et al.* 2003). It is well known that TSSM infestation decreases nitrogen, phosphorous and magnesium, as found in our investigation. These reductions may be due to reductions in their uptake and/or drain of phloem sap by TSSM. Another study has shown that *Nilaparvata lugens* infestation reduced the nutrient uptake of rice plants, particularly phosphorous (Wu *et al.* 2004).

Induced resistance to herbivores is observed in many crop plants in the laboratory and field (Creelman *et al.* 1992, Inbar *et al.* 1999). In recent years, elicitors (i.e. MeJA or SA) have been found not only to regulate a variety of plant-developmental responses, but also to be involved in regulating diverse plant functions, including induced resistance to herbivores (Thaler *et al.* 1999, Omer *et al.* 2001). JA or SA is produced by the plant after caterpillar damage and results in increased production of compounds involved in resistance (Thaler *et al.* 1996), including hydrogen peroxide, superoxide anions and hydroxyl free radicals (Devoto & Turner 2003). These observations suggest that either SA or MeJA could be linked to the

oxidative burst responsible for cell death in the hypersensitive response after pathogen or pest attack, and could act as a secondary signal for the development of systemic acquired resistance (Shirasu *et al.* 1997). Pretreatment with elicitors protects plants from oxidative damage caused by these stresses, and is often associated with an increase in antioxidant enzymes (Table 2, Daneshmand *et al.* 2009, Parra-Lobato *et al.* 2009).

MeJA is an important cellular regulator involved in diverse developmental processes, such as shoot growth, yield and seed quality (Farouk & Osman 2009). In addition, jasmonates activate plant defence mechanisms in response to insect-driven wounding, various pathogens, and environmental stress (Cheong & Choi 2003). In accordance with these results, induction of oxidative stress has been observed in plants exposed to MeJA (Ali *et al.* 2006, Sarosh & Meijer 2007). Recently, in *Arabidopsis thaliana*, the expression of oxidative stress-tolerance genes, including those for peroxidases and oxidases, was also up-regulated by MeJA treatment (Jung *et al.* 2007). However, it has been proposed that these molecules act as secondary messengers in systemic acquired resistance systems, in analogy to the systemic response induced by wounding (Ryan 1992). Reports on the induction of a resistant state in plants by MeJA are contradictory. Different effects of MeJA on protective enzyme activities could be associated with H_2O_2 metabolism (Ali *et al.* 2006). MeJA may be a suitable candidate for insect control in agriculture. No negative effects on crop yield (Farouk & Osman 2009) have been found, and plant resistance is enhanced both by directly killing herbivores and by enhancing the action of natural enemies of herbivores (Thaler 1999).

SA is an important signal molecule in plant defence. The mode of SA was proposed to be inhibition of catalase activity and increase in H_2O_2 levels during plant-pathogen interactions (Chen *et al.* 1993). In the present study, pretreatment with SA alone specifically inhibited catalase activity (Table 2) and increased H_2O_2 levels (Table 1) in bean shoots. SA can also act as an inhibitor of a step in the biosynthesis of jasmonic acid, a compound involved in wound-induced gene expression (Pena-Cortes *et al.* 1993). It remains to be seen whether these actions are of direct relevance for systemic acquired resistance. Moreover, as indicated from the present investigation, application of SA significantly increased peroxidase activity, which destroys hydrogen peroxides, leading to decreasing malondialdehyde, and improved membrane permeability. These results are similar to those of Daneshmand *et al.* (2009). Besides inhibition of catalase, SA-blocked electron flow from substrate dehydrogenisation to the ubiquinone pool in mitochondria might also be an important mechanism of triggering H_2O_2 generation (Norman *et al.* 2004). Several reports, however, show that H_2O_2 is unlikely to be a second messenger for SA (Neuenschwander *et al.* 1995). In the present study, SA treatments lowered the electrolyte leakage in TSSM-stressed plants (Table 1), concordant with Stevens *et al.* (2006) in the case of tomato, and Yildirim *et al.* (2008) regarding cucumber, who reported that SA facilitated the maintenance of membrane functions. This may be attributed to the production of antioxidant compounds and enzymes that protect the plant from oxidative damage (El-Tayeb 2005).

In plants, peroxidases are a class of proteins with a large variety of functions. They may be part of the defence system against herbivory (Trevisan *et al.* 2003), they may catalyze for instance the final polymerization step of lignin synthesis, produce hydrogen peroxide or other phenoxy radicals such as phenole that may directly deter feeding by insect herbivores, and/or produce toxins that reduce plant digestibility (Felton 1989). The present investigation showed that application of either SA or MeJA after or before TSSM infestation significantly increased peroxidase activity as compared with control or unsprayed infested plants. Our results agree with those of Mahdavian *et al.* (2007) and Daneshmand *et al.* (2009) who indicated that application of SA increased peroxidase activities in plant tissues. This increase may be attributed to increased activity of peroxidase-encoding genes or increased activation of already existing enzymes, as suggested by Dionisio-Sese & Tobita (1998). In the literature, only one work about the induction of peroxidase by exogenous MeJA was found (Garrido *et al.* 2003), stimulating $O_2^{\bullet -}$ synthesis by root cells.

As indicated from our results and other investigations, catalase levels decreased significantly following herbivory by *H. zea* (Bi & Felton 1995) and TSSM, or following exogenous application of SA. Several reports have shown that SA at high concentrations (1 mM) can bind to and inhibit several heme-containing enzymes such as catalase, ascorbate peroxidase, or aconitase by virtue of its affinity for iron (Ruffer *et al.* 1995). In the present case, a significant decrease in catalase activity was found in bean leaves in the absence of TSSM (Table 2), while SA modulated catalase activity back to the range of the untreated control in the presence of TSSM. Similar to these results, addition of 0.5mM SA to hydroponic cultures for 24 hr decreased catalase activity in maize plants (Janda *et al.* 1999). Although decreased catalase activity was observed in the results of the TSSM infestation, increased catalase activity (Table 2) was observed in the MeJA treatments, showing higher capacity for the decomposition of H_2O_2 generated. Since enzymes involved in antioxidant metabolism are usually co-regulated and their activities increase upon exposure to stress, higher activity levels in bean shoots indicate increased oxidative stress upon MeJA exposure. The observations also strongly imply a possibility that enzymatic antioxidant systems are also utilized in bean to alleviate oxidative stress caused by MeJA, thus protecting the cells from oxidative damage. Recently, Parra-Lobato *et al.* (2009) indicated that catalase activity of homogenates was higher after 30 min in MeJA-treated sunflower roots.

Application of both elicitors increased antioxidant compounds such as ascorbic acid, phenols, proline, carotenoids and soluble carbohydrates which counteracted the harmful effects of ROS on the plants. These results are consistent with the role of both SA and MeJA in defensive responses that have been extensively studied in other plants. SA and MeJA have been reported to induce anatomical and chemical changes such as the increasing synthesis of phenolics in many plants (Zeneli *et al.* 2006, Raju *et al.* 2008). This accumulation may be due to the induction of phenyl amino layase gene expression and synthesis of phenolic compounds (Raju *et al.* 2008). Yet total phenols have long been considered as important defence-related compounds whose levels are naturally high in resistant varieties of many crops (Gogoi *et al.* 2001). In this concern Raju et al (2008) found that in some plants application of SA increased phenol content due to the activation of the phenyl amino layase enzyme.

Of the antioxidants found in plants, ascorbic acid is the most abundant and has diverse physiological roles, being a substrate for ascobate peroxidase in addition to directly scavenging superoxide, hydroxyl radicals, and singlet oxygen: it is therefore an antiherbivory agent (Smirnoff *et al.* 2001). In fact, a high level of endogenous ascorbate is essential to maintain the antioxidant system that protects plant from oxidative damage due to biotic and abiotic stresses (Chen *et al.* 2007).

Proline is another important component of the defence system of plants to counter stress. It has been previously shown that proline levels increase in bean leaves in reponse to TSSM infestation or elicitor application before or after infestation. Our result corroborates those of El-Tayeb (2005), who claimed that proline could be considered an important component in the spectrum of SA-induced protective reactions of plants against salinity stress. These results are consistent with the proposal of Khattab (2005) that showed the increased proline content of eucalyptus leaves infested with xylem-feeding insects. Proline is capable of movement between tissues, and is believed to protect plant against stress by acting as a storage compound for both carbon and nitrogen sources, thus protect cytoplasmic enzymes and cellular structure (Serrano & Gaxiola 1994). Additional roles for proline such as ROS detoxification and stabilization of cell membranes have been proposed (Kavir Kishor *et al.* 2005).

Previous research has shown that most herbivorous insects use carbohydrates as feeding stimulants (Blaney & Simmonds 1988) and as important nutrients needed to synthesize body tissue and serve as energy sources (Schoonhoven *et al.* 1998). Derridj *et al.* (1989) reported that sugars have also been shown to promote oviposition in some species. Carbohydrates may directly affect mite level damage via changes in the nutritional quality of

bean plants. Such effects might be due to the drain of assimilates towards the insect and/or decreases in their biosynthetic pathways induced by TSSM. A strong and persistent flow of host assimilates is created by the continual removal of metabolites and breakdown of insoluble reserves by insects (Khattab 2005). The phloem-feeding TSSM continually controls and/or modifies the levels of metabolic substances in the surrounding tissues. This is supported in the current work where the levels of total soluble carbohydrates of infested bean leaves were lower than those of healthy ones (Table 3).

Results also showed that the application of both elicitors, in particular SA, increased significantly the ion content (N, P, K) in bean shoots, reflecting increasing plant growth and induced resistance to pests due to their role in plant metabolism, such as promoting the development of thicker outer walls and the stability of plant membranes in epidermal cells, thus preventing pest attack (Marschner 1986). Increasing nitrogen in plants stimulates the synthesis of phenols and lignin, all parts of the defence system against infestation (Marschner 1986). Earlier studies indicated that exogenous SA treatments stimulated root formation and increased mineral uptake by plants (Khan *et al.* 2003, Yildirim *et al.* 2008).

In conclusion, foliar application of both elicitors, particularly SA, enhanced enzymatic and non-enzymatic antioxidants in bean shoots infested by TSSM, thus suppressing invasion-induced oxidative damage and enhancing tolerance. Current knowledge limits the complete description of elicitor signal-transduction pathways in plants: future studies are needed to dissect the complex network of elicitors and its involvement in plant defence against biotic and abiotic stresses, using genetic, genomic and biochemical approaches.

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الملخص العربي

الملخص العربي
التغلب علي إجهاد الأكسدة الناتجة عن إصابة نباتات الفاصوليا بالعنكبوت الأحمر وذلك من خلال إستخدام المحفزات

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أدت إصابة الفاصوليا بالعنكبوت الاحمر إلي تفاقم إجهاد الاكسدة مع زيادة محتوي النبات من السكريات الذائبة، الفينولات، البرولين مع زيادة نشاط إنزيم البيروكسيديز. علي الجانب الآخر تعمل الإصابة علي إنخفاض شديد في نشاط إنزيم الكتاليز ومحتوي النبات من حامض الأسكوربيك والكاروتينيدات الكلية.
إستخدام المواد المحفزة تدفع نبات الفاصوليا علي مقاومة وتحمل الاصابة بالعنكبوت الأحمر من خلال زيادة نشاط إنزيم البيروكسيديز ومحتوي النبات من المواد المضادة للأكسدة بالتالي ينخفض محتوي النبات من فوق أكسيد الهيدروجين الذي يستتبعة قلة أكسدة الدهون والمحافظة علي نفاذية الأغشية الخلوية بالتالي المحافظة علي نسبة الأيونات داخل النبات.
من النتائج السابقة يتبين لنا أن إستخدام المحفزات تنشط الإنزيمات المضادة للأكسدة وتشجع تكوين وتراكم المواد المضادة للأكسدة بالتالي التحكم في تكوين الشوارد الأوكسيجينية الحرة مما يحسن ويحافظ علي خواص الأغشية الخلوية والجزيئات الحيوية الأخري بالتالي تحسن النمو والمحصول تحت ظروف الإصابة.