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Full Length Research Paper

Priming *Pisum sativum* with salicylic acid against the leafminer *Liriomyza trifolii*

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In a field study carried out to determine the percentages of parasitism of the leafminer *Liriomyza trifolii* attacking *Pisum sativum* (nili plantation), the parasitoid *Diglyphus isaea* was the most abundant recorded parasitoid species of the pest. In addition, a laboratory work was done to ameliorate the risk of herbivory stress by priming the plants with salicylic acid (SA) (1.5 mM) at the vegetative stage. Herbivory induced structural changes in infested leaflets including the rupture of both the upper epidermis and the palisade tissue (the photosynthetic tissue) of the infested plants. Proline is the most remarkable defense strategy in infested plant, while polyamines are in SA treated plants. Finally, it could be concluded that the natural role of the parasitoid *D. isaea* must be extensively encouraged. Also, using elicitors as salicylic acid to induce resistance of crop plants against herbivores has been successful, and seems to promise as one of the alternative pest management tools.

Key words: Parasitism, *Pisum sativum*, *Liriomyza trifolii*, herbivory, leafminers, salicylic acid, polyamines, proteins.

INTRODUCTION

Leafminers are herbivores that selectively eat only the layers that have the least amount of plant cellulose. They show non-random distributions both between and within plants (Tatiana and Peter, 2008). Initial signaling events at the plant-insect interface include stimulus-induced change in plasma membrane potential of the host and rapid changes in Ca^{2+} flux which play an important role in the control of defensive processes (Maffei et al., 2004). Moreover, plant defensive metabolites and proteins thwart herbivory by exerting direct repellent, antifeedant, and toxic effects on the insect. Synergistic interactions between these compounds strengthen the host defense response (Amirhusin et al., 2007). Primed plants display either faster, stronger or both activations of the various cellular defense responses that are induced following the attack by insects or in response to abiotic stress. Effects of priming compounds such as, salicylic acid may be mediated through effects on plant defense chemistry or other aspects of the suitability of foliage for insect feeding and growth (Sarwar et al., 2008).

P. sativum represents one of the most important popular foods, having high nutrients values of human consumption, either as green pods or dry seeds. This crop is liable to be attacked by several insect pests from the early stage of growth through late development to the harvest stage. The leafminer *L. trifolii* was shown as one of the most destructive insect pests attacking *P. sativum* plants (Jyani et al., 1995). So, the aim of this work was to focus on the role of the most important pest parasitoids and also to study the anatomical, physiological and biochemical changes accompanying induced resistance in *P. sativum* against the leafminer *L. trifolii* using salicylic acid (SA) priming.

MATERIALS AND METHODS

Field study

Survey of L. trifolii population and its parasitism

Studies were carried out at Qaluobyia Governorate, Egypt, during the nili plantation of *P. sativum* of season 2009. An area of one feddan was chosen at Shabein El-Kanater district, which received all the regular recommended agricultural practices, except absence of chemical insecticides. At the age of 14 days (at the third week of October, 2009), samples of 500 leaflets (5 leaflets/plant × 100 plants), were randomly weekly collected. Leaflets were directly transferred to the laboratory in paper bags for investigation. Collected leaflets were put in glass jars (17 cm height × 11 cm diameter), which were daily provided with filter paper (to absorb any extra moisture) until pupation or the emergence of adults of *D. isaea* parasitoids.

Emerged *D. isaea* parasitoids were carefully identified kindly in the Biological Control Research Department in the Agricultural Research Center and counted by the aid of a stereomicroscope for estimating the weekly percentages of parasitism of *L. trifolii*, according to the equation described by El-Khawas and El-Khawas (2005). Inspection of samples ended in May (in the last week of December that is, at time of harvesting).

Anatomical, physiological and biochemical studies

Plant material and induction treatments

An experiment was performed in the greenhouse of Botany Department Faculty of Science, to evaluate the effect of salicylic acid (SA) on the leafminer *L. trifolii*. Seeds of *P. sativum* were obtained from Agricultural Research Center at Giza, where this tested host plant was planted in individual plastic pots of 20 cm diameter containing a mixture of peat and sand (1:1). All plants were grown in normal natural condition. Plants were arranged in three groups (groups 1, 2 and 3). Each set consisted of twenty pots, and five plants were maintained in each pot. At the vegetative stage (2 weeks old), group 1 served as healthy plants that is, without infestation by the pest, group 2 served as infested plants without any treatments with salicylic acid and were sprayed with water only, while group 3 served as infested plants that was sprayed with 1.5 mM salicylic acid for inducing resistance against the leafminer *L. trifolii*. However, collected infested *P. sativum* leaflets were brought to the laboratory for rearing of the leafminer *L. trifolii* for the experimental purpose. The rearing technique was done following the technique described by Rathman et al. (1991). The physiological and biochemical effects on *P. sativum* plant leaflets under herbivory by *L. trifolii* were evaluated. The following was estimated to compare the effect of spraying salicylic acid on infested *L. trifolii* plants (group 3) in relation to the other two groups (1 and 2).

Estimation of pigments

Chlorophyll a and b, and carotenoids contents were determined according to Saric et al. (1976).

Estimation of sugars

Total soluble sugars were extracted following the method adopted by Homme et al. (1992) and determined with the anthrone reagent (Whistler, 1962).

Phenol content

Phenol content was estimated in the ethanol extract using Folin-Ciocalteau phenol reaction (AOAC, 1990).

Metal ions determination

Samples were digested in nitric-perchloric acid mixture (Miller, 1998) and analyzed with an atomic adsorption spectrometer where they were analyzed in triplicates.

Estimation of nitrogen

Total nitrogen was determined in the acid digested samples by the conventional micro-Kjeldehl method as described by Yemm and Willis (1956).

Estimation of proline

Free proline was determined according to the technique described by Troll (1995).

Estimation of polyamine content

A certain weight of shoot plant sample was homogenized in a Waring blender in 5% perchloric acid (PCA), according to the method described by Shalaby (1995). Polyamine [putresine (put) $\{H_2N\ (CH_2.NH_2)\}$, spermidine (spd) $\{H_2N.(CH_2)_3.NH.(CH_2)_4.NH_2\}$ and spermine (spm) $\{H_2N.(CH_2)_3\ (CH_2)_4NH.CH_2\}_3]$ levels were determined, using high performance liquid chromatography as described by Smith and Davies (1985).

Protein analysis

Soluble proteins were determined according to the method described by Bradford (1976). Eectrophoretic protein profile was analyzed by SDS-Page technique (Laemmli, 1970). Data were analyzed and identified by gel documentation system (GDS) with standard markers using Gel Proanlyzer Version 3 Media Cyberene Tice Imaging Experts Software.

Protective enzymes

Catalase (E.C.1.11.1.6) activity was assayed according to Aebi (1983), where decomposition of H_2O_2 is followed spectrophotometrically at 240 nm. One unit of enzyme activity is equal to 1 μmol of H_2O_2 decomposed per min. Polypehenol oxidase (PPO, EC 1.10.3.1) activity was measured using the method of Malik and Singh (1980). Chlorophyllase enzyme was extracted by using a modification of the method of Fernandez-Lopez et al. (1992).

The anatomical and cellular changes

To examine the anatomical and cellular changes which occurred in the infested leaflet compared with the healthy and treated ones, transverse leaf sectioning was made. The free-hand sections were stained using safranine and light green for microscopic examination.

Statistical analysis

The obtained data were statistically analyzed according to procedures outlined by Snedecor and Cochran (1980) and the least significant differences (L.S.D.) test was run to compare the mean values at 1 and 5%, using SAS Program (1994).

The means of temperature and relative humidity were obtained from the Meteorological Station of Agricultural Research Center (ARC).

RESULTS AND DISCUSSION

Survey of *L. trifolii* populations and its parasitism

Data obtained in Table 1 showed that, the infestation of

Table 1. Total numbers of *L. trifolii* larvae and the percentages of parasitism by the parasitoid *D. isaea* during nili plantation of *P. sativum* of season 2009.

Date of samples	Total number of pest larvae	Total parasitism (%)	Mean temperature (°C)	Mean R.H (%)
21/10/2009	0	0	24.9	49
28/10/2009	3	0	21.1	49
11/4/2009	11	9.09	26.4	48
11/11/2009	47	29.79	16.6	47
18/11/2009	91	50.55	17.3	47
25/11/2009	59	33.9	16	47
12/2/2009	37	24.32	16.5	49
12/9/2009	23	17.39	13.8	51
16/12/2009	10	10	12.1	50
23/12/2009	2	0	11.8	49
Mean/season	28.30 (0-91)	17.50% (0.00-50.55)	17.7(11.8-26.4)	48.6% (47.0-51.0)

Table 2. Changes in the mineral composition of leaflets of *P. sativum* against herbivory by *L. trifolii* .Values listed are expressed as mg/g Dwt. Each value is a mean of three replicates.

Samples	K	Ca	P	Mg	Fe
Healthy (control)	2.60	2.20	0.22	33.50	200
Infested	2.30**	1.80**	0.19*	24.10**	180**
SA treated	3.20**	3.30**	0.22	33.50	270**
LS.D. at 1%	0.020	0.005	0.001	1.600	3.100
L.S.D. at 5%	0.040	0.007	0.003	2.100	7.200

**Highly significant differences, *significant differences.

P. sativum plants by *L. trifolii* was at the maximum numbers (91 larvae/500 leaflets) during the third week ofNovember, 2009. The parasitoid, *D. isaea* (Walk.) (Hymenoptera: Eulophidae), was the most abundant recorded parasitoid species of the pest in nili plantation; where the highest percentage of parasitism (50.55%) of the parasitoid was synchronized with that of the pest. Data obtained in Table 1 was explained by Awadalla and Fathy (1995) who stated that, the parasitoid *Diglyphus* sp. seemed to be the most common and efficient parasitoid species attacking *L. trifolii* on cowpea plants, being represented throughout the longest period and in the highest percentages of parasitism.

Physiological and biochemical studies

It was obvious from Figure 1 that, herbivory induced structural changes in infested leaflets including the rupture of both the upper epidermis and the palisade tissue (the photosynthetic tissue) of the infested plants. Leafminers can elicit a formation of mines which result from the feeding and movement of the larvae on the upper leaflet surface. In this connection, extensive leaf mining of *Aesculus hippocastanum* L. by the larvae of an invasive moth (*Cameraria ohridella Deschka* et Dimic) destroyed the palisade tissue and leaves, and showed seasonal changes in hydraulic resistance which were related to ontogeny (Nardini et al., 2010). In this study, insect herbivory induces significant reduction in the levels of magnesium, iron, potassium and calcium of *P. sativum* (Table 2). In contrast to the above results, exogenous SA significantly increased such levels and the activities of these ions. In accordance with these results, Orcut and Nilsen (2000) stated that the reduction in mineral content may be due to the decrease in the ion uptake; herbivory causes imbalance nutrient uptake, leading to altering the enzyme activities and consequently disorder the cell metabolism.

Chlorosis of *P. sativum* leaves which results from leafminers attack may be explained by the significant reduction in chlorophyll a and b levels, as well as the significant increase in carotenoids observed in the infested leaves as compared with the healthy ones (control and SA treated plants) (Table 3). The decrease in the photosynthetic pigments may be attributed to theinhibition of pigments biosynthesis due to Mg deficiency which is a constituent of chlorophyll or due to damage of palisade tissue which contains the chloroplast. Furthermore, the degradative function of chlorophyllase in Table 3 may explain both leaf chlorosis and drop in chlorophyll content and consequently, damage of pest

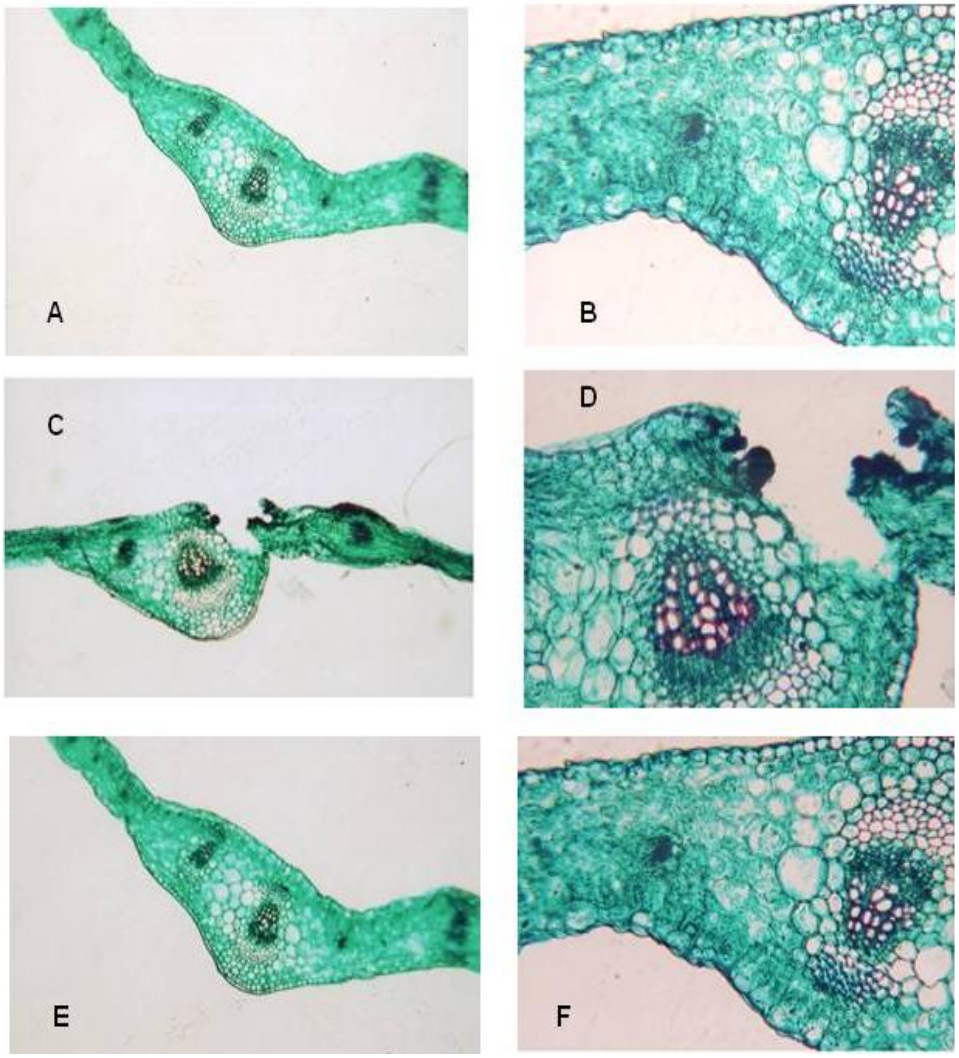

Figure 1. Free hand transverse sections of leaflets of *P. sativum* showing the structural changes in the palisade tissues. A: control, B: control (enlarged), C: infested, D: infested (enlarged), E: SA treated, F: SA treated (enlarged).

Table 3. Changes in the photosynthetic pigment, chlorophyllase activity and carbohydrate contents of leaflets of *P. sativum* against herbivory by *L. trifolii.* Each value is a mean of three replicates.

Sample	Photosynthetic pigment(Ug/100 g f.wt.)				Chlorophyllase	Sugar metabolites(mg/100 g f.wt.)	
	Chl a	Chl b	Chl a+b	Cartenoids	Umol/min	Soluble sugar	Total carbohydrates
Healthy (control)	46	26	72	30	2.0	3.22	24.24
Infested	25**	16**	41**	42**	6.0**	1.89**	13.20**
SA treated	92**	63**	155**	35	1.9	4.62**	28.10
L.S.D. at 1%	3.4	4.6	9.4	3.8	0.7	0.04	4.3
L.S.D. at 5%	5.7	5.1	9.8	6.7	1.2	0.09	6.2

**Highly significant differences, *significant differences.

attacks on plants would be through reducing photosynthetic activity in their hosts. In accordance with this view, Yemm and Willis (1956) reported that the chlorotic symptoms observed on insect infested wheat may be elicited by unbalanced chlorophyll biosynthesis and degradation.

Table 4. Changes in proline, polyamines, total soluble protein, total nitrogen ,total phenol ,catalase and polyphenol oxidase contents of leaflets of *P. sativum* against herbivory by *L trifolii.* Each value is a mean of three replicates.

Treatment	Proline (ug/100 g fwt)	Polyamine (μg/gf wt)				Totalprotein (ug/100 g fwt)	Total solublenitrogen (mg/100 gfwt)	Totalphenol (mg/100 g fw)	Catalase Umol/min	Polyphenol-oxidase Umol/min
		Put	Spd	Spm	Total(PA)					
Healthy (control)	9	17	2.36	360	379.36	24.25	72	22	9	8
Infested	20**	12.0**	1.28**	330**	343.2	16.24*	38**	40**	8**	11.0**
SA treated	15**	22.6**	3.80**	381**	407.4	33.52**	98**	33**	12**	10.2*
L.S.D. at 1%	0.04	0.9	0.02	6	-	4.2	3.9	1.6	0.3	1.5
L.S.D. at 5%	0.09	1.8	0.05	7. 20	-	5.4	4.8	2.4	0.6	2.6

**Highly significant differences, *significant differences.

The reduction in potassium uptake is one of the factors which lead to reduced protein synthesis (Table 4). Data in Table 4 demonstrate highly significant increment in the proline content of infested plant as being compared with both the healthy and SA treated plants. Herbivory was recorded to reduce the nitrogen pool. It is suggested that increment in the proline content of infested plant as being compared with both the healthy and SA treated plants is the most obvious defense strategy. Prolines and hydroxyprolines in peptide chains are linked through imino nitrogens and C bonds that form kinks in the backbone, allowing limited conformations that impose stresses on secondary structures (Reiersen and Rees, 2001). Changes in the polyamine level in pea leaflets are recorded in Table 4. Putresine, spermidine and spermine were detected in control plants, infested and SA treated plants.

Recently, little work was done to study the relation between polyamine and biotic stress (Sagor et al., 2009). SA could alleviate the harmful effect of herbivory on pea plants by increasing the putresine content as being compared with the control plants. Polyamines such as spermine are proposed to play a role during biotic stress responses (Maike et al., 2008). The ameliorative effect of SA on the plant growth inhibition may be due to the highly significant increase in the polyamine contents (Table 4), this suggestion were supported with the results of the present work that the increase in endogenous polyamine contents was accompanied by increases in chlorophyll contents and total carbohydrates in pea plants exposed to herbivory and treated with SA. Evidences showed that polyamines are involved in many physiological processes. The exogenous application of PAs is also another option for increasing the stress tolerance potential in plants (Gill and Tuteja, 2010). Polyamines are ubiquitous polycations with pleiotropic biochemical activities, including regulation of gene expression, cell proliferation and modulation of cell signaling. Reports that the polyamines with cytoprotective activities were induced by diverse stresses raised the hypothesis that polyamines may play a role in inducing stress response. Polyamines can function as primordial stress molecules in bacteria, plants and mammals, and may play an essential role in regulation of pathogen-host interaction (Rhee et al., 2007).

Biotic stress would act upon plant development by modifying the plant protective roles, especially the secondary metabolism (Hopkins and Hüner, 2004). Estimation of the total phenols showed highly significant increments in the phenolic content of infested host plants and SA treated plants. High levels of polyphenol oxidase and catalase were detected in infested leaves as being compared to the non-infested control (Table 4). Phenolic compounds are particularly attractive as prophylactic agent in the management of ROS-mediated disorder due to their pleiotropic effects, free radical scavenging, metal chelating, antioxidant and modulation of cell signaling pathways (Soobratte et al., 2008).

Significant increments in the phenolic content of infested host plants and SA treated plants may be correlated with polyphenol oxidase (Table 4). Polyphenol oxidase (PPO) enzymes are responsible for the typical browning of plant extracts, and damage of tissue is caused by spontaneous polymerization and cross linking of o-quinones. During chewing, the mixing of (PPO) and phenolic substrates generates o-quinones and the highly reactive compounds are then able to covalently modify free amino acids and

Table 5. Protein profile and molecular weight (M. Wt.) of leaflets of *P. sativum* against herbivory by *L. trifolii*.

Band number	Marker (kDa)	M. Wt. (kDa)	Lane 1 (Healthy) (control)	Lane 2 (Infested)	Lane 3 {salicylic acid (SA) treated}
1	116	116	-	-	-
2		114	-	-	-
3		108	-	-	+
4		101	-	-	-
5	98	98	-	-	-
6		95	+	-	+
7		84	+	-	+
8		79	-	-	+
9	66	66	+	+	+
10		64	+	+	+
11		58	-	-	-
12		48	+	+	+
13		40	+	+	+
14	37	37	+	+	+
15		33	+	+	+
16		22	+	+	+
17		19	+	+	+
Total			10	8	12

M: Marker, +: present, - : absent.

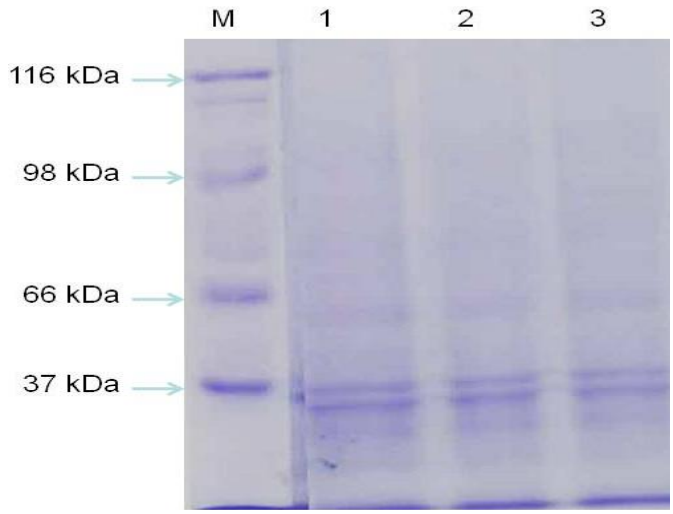

Figure 2. Protein profile of leaflets of *P. sativum*. Lane M: protein molecular weight marker, lane 1: healthy (control), lane 2: infested, lane 3: SA treated.

sulfhydryl groups in dietary protein within the mouth and gut of the insect (Bellucci et al., 1999). However, Constabel et al. (2000) stated that, the resulting phenolic adducts prevent efficient assimilation of the alkylated amino acids and thus reduce the nutritive value of protein. High levels of polyphenol oxidase and catalase in Table 4 may have important functions in scavenging of reactive oxygen species (ROS) during plant metabolism. Data recorded in Table 5 and Figure 2 showed significant reduction and increment in infested and SA treated plants as being compared to the healthy control. The most valuable remark is the appearance of 2 new inducible protein bands in response to SA treatment (108 and 79 kDa), another two bands are common between control and SA treated plants (95 and 84 kDa). The protein bands of low molecular weight ranged between 48 and 19 kDa, and are common for all samples. As an explanation of the above result, the gene product of being a 33 kDa cysteine proteinase inhibits the growth of a wide range of lepidopteron larvae (Pechan et al., 2000). The polypeptide 48 kDa may be related to dehydrines that may interact with compatible solutes to serve as structural stabilizers of macromolecules. Moreover, Kandoth et al. (2007) stated that MAPKs, LeMPK1, LeMPK2, and LeMPK3 function in the systemin-mediated defense response against herbivorous insects. Insect feeding triggers the expression of plant defensive proteins that exert direct effects on the attacker. PIs (which impair various mechanistic classes of digestive proteases in the insect midgut) results in amino acid deficiencies that negatively affect the growth and development of the herbivore (Lison et al., 2006). The effectiveness of PIs as a defense is often thwarted by the insect's adaptive ability to express digestive proteases that are insensitive to the host plant complement of PIs, or that inactivate PIs (Bayes et al., 2005). The plant's defensive protein arsenal also includes enzymes that

disrupt insect digestive physiology and other aspects of food consumption. Members of the cysteine protease family of enzymes disrupt the chitin-rich peritrophic membrane that protects the gut epithelium (Mohan et al., 2006). A future work can be done to detect the volatile emitted in response to priming and on the molecular level, and also to detect the specific gene or genes involved in such process.

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