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Differential Effect of Jasmonic Acid on the Defense of Faba Bean Against *Fusarium* Wilt: Changes in Protein and DNA Patterns, Peroxidase and Esterase Isozymes

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ABSTRACT

Seed treatment with different concentrations (5, 10 & 20 mM) of jasmonate (JA) or a potent fungicide (Rhizolex-T) resulted in obvious modulations in the protein patterns of faba bean plants (30-day-old) infected with *Fusarium oxysporium* f.sp. *fabae*. The most observed change was the progressive decrease in the number of protein bands with the increase of JA concentration and farther decrease by the fungicide, in the infected plants. In the non-infected plants, similar treatments did not approximately change the number of protein bands. The effect of the fungicide mimicked that of JA, particularly at 10 mM, under the influence of the fungal infection. Variation in the genomic DNA was also investigated, using arbitrary decamer primers**.** The changes in DNA fragments, with respect to different treatments, were also more evident in the infected than in the non-infected plants. Modulations of DNA sequences showed similar trends to those shown with corresponding protein patterns. The above mentioned effects of JA and the fungicide were also concomitant with enhanced number and concentration of peroxidase and esterase isozymes.

Key Words: Faba bean; *Fusarium* Wilt; Jasmonic acid; Fungicide; Protein patterns; DNA

INTRODUCTION

Jasmonate family of signaling molecules includes jasmonic acid (JA), its methyl ester (MeJA), and their metabolic C18 precursor, 12-oxo-phytodienoic acid (Rayan, 2000). Jasmonates are synthesized from linolenic acid via the octadecanoid pathway (Schaller, 2001). Increases in JA in response to pathogen attack occur both locally and systemically and a subset of inducible plant defense genes from *Arabidopsis* require a JA-dependent, salicylic (SA)-independent signaling pathway (Hammond-Kosack & Jones, 2000). In addition, JA and ethylene are required for activation of proteinase inhibitor (PI) genes and certain pathogenesis-related (PR) genes (Staskawicz *et al.*, 1995). Chitinases (Pan *et al*., 1999) and glucanases (Grenier *et al*., 1999) represent important PR proteins that degrade structural polysaccharides of fungal cell walls and mostly reduce fungal growth. Other defense proteins (or peptides) such as lipoxygenase may contribute to defense by generating secondary signal molecules, as JA and lipid peroxides, and by producing an array of toxic secondary metabolites with substantial antimicrobial activity (Veronese *et al.*, 2003). Defensins (a family of basic cysteine-rich peptides with a molecular mass of less than 7 kDa) are also important proteins having antimicrobial activity. Their signaling pathway, in vegetative plant tissues, is mediated by JA and ethylene, not SA (Terras *et al*., 1995). A plant defensin encoded by the *PDF1.2* gene of Arabidopsis is commonly used as a marker for characterization of the jasmonate-dependent defense responses (Brown *et al*., 2003). Expression by JA not only enhanced resistance against fungal and bacterial pathogens (Gu *et al*., 2002), but also conferred tolerance to abiotic stresses; e.g. osmotic stress (Park *et al*., 2001).

Production of reactive oxygen species (ROS) in plants is well documented and a key component in plant defensive responses (Lamb & Dixon, 1997). Recent studies provide evidence that ROS function downstream of JA to amplify wound and systemin-induced responses (Li *et al*., 2001).

Numerous Peroxidase isozymes exist in plants and are mainly localized in the cell walls and vacuoles (Carpin *et al*., 1999). Peroxidase isozymes interfere with jasmonate signals, since they can be induced by jasmonates (Curtis *et al*., 1997), wounding (Bernards *et al*, 1999), and pathogenesis (Lamb & Dixon, 1997). They are also included, with phenylpropanoids, in the synthesis of lignin (Whetten *et al*., 1998) and mediation of cross linking of polysaccharides or extensions of the cell wall, thus serving in the wall construction and regulation of cell wall plasticity (Browleader *et al*., 2000; Takeda *et al*., 2003).

Esterase isozymes might also interfere in JA-related processes via hydrolyzing methyl jasmonate, methyl esters of ABA, and fatty acids (Stuhlfelder *et al*., 2002).

The effect of JA and a specific fungicide (Rhizolex-T) on the defense of faba bean against *Fusarium oxysporium* f.sp. *fabae* wilt fungus from the point of view of modulation of phytohormones (other than jasmonates) and phenolics has been studied previously (Ahmed *et al*., 2002). Thus, the

present work was devoted to investigate the effects of seed treatment of faba bean with different concentrations of JA and the fungicide on the patterns of protein and DNA, as well as peroxidase and esterase isozymes in *Fusarium*-infected and non-infected plants.

MATERIALS AND METHODS

A pure cell line of faba bean (*Vicia faba* var. Giza 716) was obtained from the Agriculture Research Center, Ministry of Agriculture, Cairo, Egypt. Jasmonic acid (JA) was brought from Sigma-Aldrech Company. Rhizolex-T (Tolclofos methyl thiram) was brought from Sumitomo Chemical Company Ltd.. Seeds were sterilized by 10% sodium hypochlorite. Treatments were given by soaking the seeds for 8 h in solutions of JA (5, 10, or 20mM), the fungicide Rhizolex-T (500 ppm), or in water (control). Pots (30-cm diam.) were filled with constant amounts of sterilized sandy loam soil (1:2 w/w). The prepared group (40 pots) was divided into two equal subgroups, each divided into five sets (control H_2O, JA: 5, 10 & 20 mM, & the fungicide treatments). Sowing was carried out in the first subgroup in sterilized soil, whereby in the second, soil was infected with inoculum (5% of soil weight/pot) of *Fusarium oxysporum* f. sp. fabae. Irrigation of all pots was carried out routinely using a constant amount of tap water per pot. Pots were placed under natural conditions of daily light (11 h) and darkness (13 h), where the temperature varied from 20±2°C during the day to 10±2°C at night. The relative humidity varied from 60 to 80%. After 30 days, the plants were collected for different analysis.

Extraction, separation, and gel scanning of proteins. Extraction of protein was carried out in fresh leaves of 30-day-old plants. The tissue was ground to a fine powder with liquid nitrogen. Half gram powdered tissue was mixed with one and half ml Tris-HCl buffer at pH 8.5, vortexed and left overnight at 4°C. The extract was then centrifuged at 6000 rpm for 20 min and the supernatant transferred to a fresh tube. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used according to Dunn (1993) and preparation of the slab gel (15%) and protein samples was followed as described by Hames (1981). Protein samples (20 µg) were loaded together with BioRAD molecular markers (113-20 kDa). Destained gel was photographed and quantitative analysis of protein patterns was done using a laser gel documentation system.

Extraction and PCR amplification of genomic DNA. DNA was extracted from 1 g fresh tissue of leaves (30-day-old plants), as described by Paterson *et al.* (1993). The extracted DNA was then used as an amplification template for RAPD-PCR analysis, using four arbitrary decamer primers (Operon Tech. Inc., Kit A, USA). The nucleotide sequences and guanine-cytosine (GC) ratios of the used primers are shown in the following table:

Primer Code	Sequences (5 →3)	% GC
OPA-01	CAGGCCCTTC	70
OPA-02	TGCCGAGCTC	70
OPA-03	AGTCAGCCAC	60
OPA-04	AATCGGGCTG	60

The total reaction volume was 50 µl containing 100 ng DNA, 50 pmol primer, 200 µM $DNTP_4$ (dATP, dGTP, dCTA, & dTTP), 3 mM $MgCl_2$, and 0.5 Tag polymerase. Amplification was performed using DNA thermal cycler (Perkin Elmer Cetus, model 2400). The thermal conditions were as follows: 6 min at 94°C (hot start), 40 cycles (1 min at 94°C, 2 min at 36°C, & 3 min at 72°C), and a final extension (post extension) of 10 min at 72°C. The PCR products were run out on 1.8% agarose gels and then stained with ethidium bromide. Separation was documented by photography, using Polaroid^R camera. The values of DNA fragment sizes (bps) as well as those of the DNA markers were automatically computerized.

Electrophoresis of isozymes. Leaf samples (0.5g) from 30-day-old plants were ground with liquid nitrogen in a morter 1.5 ml extraction buffer (Tris-borate: 0.125 M at pH 8.9) to isolate esterase and peroxidase isozymes. Samples were transferred to fresh tubes and kept at 4°C for half an hour. Then samples were centrifuged at 12000 rpm and 4°C for 20 min. The supernatants were stored at 20°C until native PAGE was performed to fractionate the isozymes according to Stegemann *et al.* (1985). A volume of 40 µl extract of each sample was mixed with 10 µl glycerol and 10 µl bromophenol blue. Then, 50 µl of this mixture was loaded on the gel for peroxidase, 40 µl for esterase isozymes. The method adapted by Larsen and Benson (1970) was followed for appearance of the bands of peroxidase, whereby that of Scandalios (1964) was followed for those of esterase. Then, the gels were photographed and diagrammed.

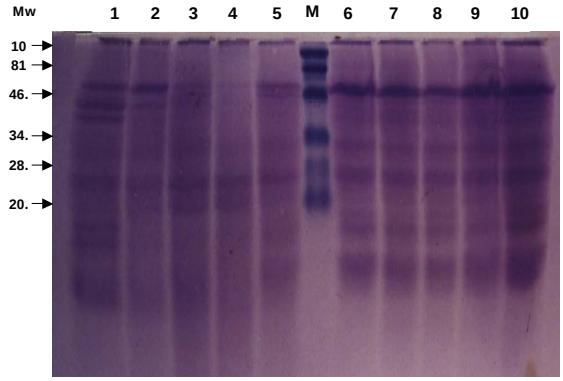
RESULTS AND DISCUSSION

Protein patterns. Fig. 1 shows the protein banding patterns in the extracts of the control (H_2O), fungicide-treated plants, as well as those treated with JA (5, 10 & 20 mM) in prevalence or not of the fungal infection. A computer scanning further clarified the different protein bands and their relative concentrations, where the results are shown in Table I. These results indicate the occurrence of 10 protein bands in the control (H_2O) of either the non-infected or the fungus-infected plants. In the fungus-infected plants, the number of protein bands decreased from 11 to 7 with the increase of JA concentration from 5 to 10 & 20 mM. On the other side, the number of protein bands in the non-infected plants was comparable and irrespective of JA concentration. In response to treatment with the fungicide in the infected plants, the protein patters were more or less similar to those in treatments with the higher concentrations of JA. In the non-infected plants, the number of protein bands was comparable in the fungicide-treated and different JA-treated plants.

Table I. Effect of jasmonic acid at 5mM (JA1), 10 mM (JA2), 20 mM (JA3) and the fungicide Rhizolex-T on the protein-banding patterns of faba bean plants (30-day-old), infected and non-infected with Fusarium wilt

Mw KDa	Infected					Non-Infected					Sum
	JA_1	JA_2	JA_3	Fungicide	Control	JA_1	JA_2	JA_3	Fungicide	Control	
					%						
54	13.05	9.50	9.27	7.55	9.54	11.03	9.75	8.78	9.32	12.20	100
45						22.76	21.00	18.44	20.03	17.78	100
41	15.20	10.41	10.59		10.34	11.29	10.19	9.02	9.83	13.13	100
40	19.10				13.05	14.36	13.00	11.48	12.52	16.50	100
35	100.00										100
32	13.94	9.75	10.19	9.00	6.66	10.66	9.46	8.72	9.26	12.36	100
24	17.18	11.94	12.65	11.69		13.05	11.59	10.65	11.26		100
20.2	13.22	9.19	6.47	11.07	14.67	9.41	8.46	7.84	8.31	11.37	100
18	17.43				12.18	12.28	10.82	10.17	10.55	26.58	100
15	26.41				16.98	11.86	10.79	9.91	10.10	13.94	100
10.5	13.68	9.34	9.95	9.02	9.87	10.15	9.03	8.27	8.84	11.84	100
9	14.35	9.72	10.33	9.18	9.83	9.61	8.93	8.05	8.59	11.43	100
No. of Bands	11	7	7	6	9	11	11	11	11	10	

Fig. 1. Electrophoretic pattern of protein bands of faba bean plants (30-day-old). Lanes 1-5, represent plants infected with Fusarium wilt [1(5 mM), 2 (10 mM), 3 (20 mM) jasomic acid, 4 (fungicide), 5 (control H_2O)]. Lanes 6-10, represent non-infected plants [6 (5 mM), 7 (10 mM), 8 (20 mM) jasmonic acid, 9 (fungicide), 10 (control H_2O)]. Relative molecular weights of electrophoresed protein standards (M) are indicated on the left



In the infected plants, the envisaged progressive repression of certain proteins with increase of JA concentration, and farther effect by the fungicide (Table I; Fig. 1) might reflect one of the strategies of these treatments to reinforce faba bean defense against *Fusarium* wilt. Plant defense mechanisms are known to be induced by pathogen infection (Veronese *et al*., 2003), microbial products in non-host and host resistance (Kamoun, 2001), and other signaling molecules including JA. In such cases, repression or induction of a number of regulatory proteins and transcription factors are implicated to enhance plant defense responses in different ways (Staskawicz *et al*., 1995; Terras *et al*, 1995; Memlink *et al*., 2001; Gu *et al*., 2002).

It is also obvious from the results in Fig. 1 and Table I that a 45 kDa protein was repressed as a result of the fungal infection, since this protein occurred in the control, JA-, and fungicide treated uninfected plants, but was absent in these plants under fungal infection. The data obtained in the present work (Fig. 1; Table I) also show a disappearance of 40, 18, and 15 kDa proteins in the infected plants, in response to treatments with the moderate (10mM) and the high (20mM) rates of JA as well as the fungicide. This might be also related to defense responses in the host, induced by the above mentioned treatments, possibly for reprogramming of cellular metabolism (Chen & Chen, 2002). This assumption might be in alliance with our previous work (Ahmed *et al*., 2002), where the same treatments (10, 20 mM JA, & the fungicide) were markedly more effective in the resistance of faba bean plants to the fungal infection than 5mM JA.

Another point of interest is the occurrence of a 24-kDa protein in the differently treated infected and non-infected plants (Fig. 1; Table I). This protein was absent, only in the control (H_2O) plants. Thus, it appeared likely to represent a unique marker of JA and fungicide treatments in prevalence or absence of the fungal infection. However, this protein might represent a homology with a jasmonate inducible protein (JIP) of 23 kDa, encoded in in barley (Hause *et al*., 1996, 2002). The induction of a 24kDa protein by also the fungicide, in our work, might further substantiate a shared process(s), either directly or indirectly, within the signal transduction pathway of JA. A plant defensin encoded by the *PDF1.2* gene of *Arabidopsis* is commonly used as a marker for characterization of the jasmonate-dependent defense responses (Brown *et al*., 2003). In a previous work (Ahmed *et al*., 2002), we have found that abscisic acid (ABA) levels were markedly enhanced in roots and shoots of faba bean plants with respect to treatment with the fungicide or JA. However, there is a good deal of evidence that the plant defense signal trasduction net-work can cross-talk with other plant stress-response pathways (Park *et al*., 2001;Veronese *et al*., 2003), which are known to include ABA signal.

Changes in DNA. The results obtained (Fig. 2) generally indicate the following:

1. In the infected plants, exogenous application of the different concentrations of JA was concomitant with the occurrence of two unique DNA fragments that were not observed neither in the corresponding control nor in the other treated plants. These fragments exhibit approximate sizes (bp) of 9069, with the primer OPA-2, and 1733, with OPA-3. Many authors document a JA-mediated activation of a number of genes and transcription factors. For example, JA was found to interfere with the regulation of the cis-acting sequence known as the GCC-box which is found in the promoters of many pathogen-responsive genes as *PDF1.2*, *Thi2.1*, and *PR4* (Brown *et al*., 2003). An ERF (ethylene response factor) subfamily of transcription factors called octadecanoid- responsive *Catharanthus* (Orca) has been implicated in JA-regulated expression of secondary metabolic pathways in *Catharanthus roseus.* Treatment with JA and fungal elicitors rapidly induced transcript level of the transcription factors called octadecanoid-responsive *Orca2* and *Orca3*. The resulting ORCA2 and ORCA3 proteins regulate overlapping at distinct sets of genes associated with secondary metabolism via specific binding to a promoter element called the jasmonate- and elicitor-responsive element, which contains a core of GCC-box (van der Fits & Memlink, 2000). The *PDF1.2* gene of Arabidopsis encoding a plant defensin is commonly used as a marker for characterization of the jasmonate-dependent defense responses (Brown *et al*., 2003). Activation of defense gene expression by JA was found to enhance resistance against fungal and bacterial pathogens (Gu *et al*., 2002) as well as resistance to abiotic stresses; e.g. osmotic stress (Park et *al*., 2001).

2. The fungicide treatment showed different effects in the infected and the non-infected plants. Thus, certain DNA fragments (153 bp with OPA-1; 372 bp, OPA-2; 990 bp, OPA-3; & 222bp, OPA-4) were observed in the infected plants and specific different ones (8485 bp, 6720 bp, & 5149 bp with OPA-1) were shown in the non-infected plants. This result might be in alliance with our previous results (Ahmed *et al*., 2002), which indicated different modulations of endogenous phytohormones in the fungus-infected and non-infected faba bean plants. The present results concerning protein patterns (Fig. 1) also delineated variation of the fungicide effect in presence and absence of the fungal infection.

Fig. 2. PCR-RAPD analysis of genomic DNA of faba bean plants (30-day-old). Lanes 1-5, represent plants infected with Fusarium wilt[1 (5 mM), 2 (10 mM), 3 (20 mM) jasomic acid, 4(fungicide), 5(control H2O)]. Lanes 6-10, represent non-infected plants [6(5 mM), 7 (10 mM), 8(20 mM) jasomic acid, 9 (fungicide), 10 (control H_2O)]. Four decamer primers (OPA: 01-04) were used. DNA fragment sizes (bp) are compared

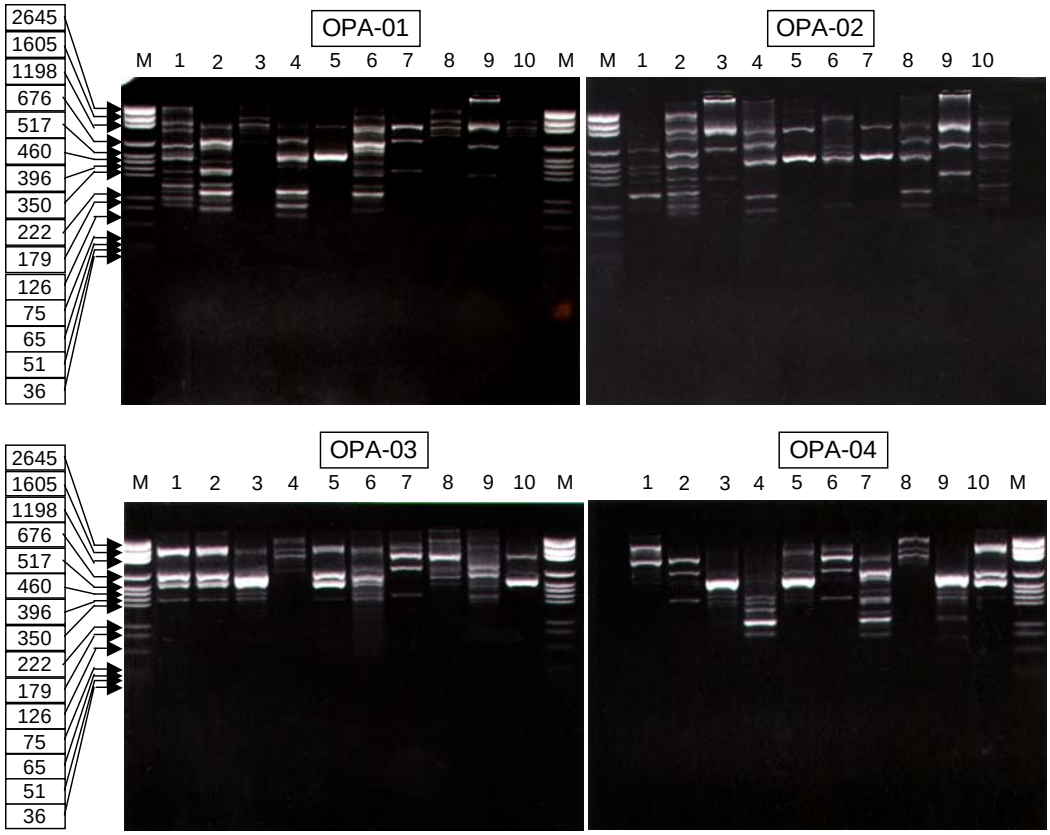


Table II. Peroxidase isozymes in shoots of faba bean plants (30-day-old), infected and non-infected with Fusaium wilt. In both cases, treatments were carried out using 5 mM (JA1), 10 mM (JA2) and 20 mM (JA3) jasmonic acid and the fungicide Rhizoliex-T. Results are expressed as % of optical density

Band No.	R_f	Infected					Non-Infected					Sum
		JA_1	JA_2	JA_3	Fungicide	H_2O	JA_1	JA_2	JA_3	Fungicide	H_2O	
		%					%					
1	0.04	10.23	12.02	12.02	7.69	8.76	8.28	5.75	13.60	9.40	12.24	100
2	0.20	5.37	9.74	9.74	7.55	6.72	8.25	7.47	19.49	13.75	11.93	100
3	0.70	57.36	42.64									100
4	0.80			55.24	44.76							100
No. of bands	4	3	3	3	3	2	2	2	2	2	2	

Table III. Esterase isozymes in shoots of faba bean plants (30-day-old), infected and non-infected with Fusaium wilt. In both cases, treatments were carried out using 5mM (JA1), 10 mM (JA2) and 20 mM (JA3) jasmonic acid and the fungicide Rhizoliex-T. Results are expressed as % of optical density

Band No.	R_f	Infected					Non-Infected					Sum
		JA_1	JA_2	JA_3	Fungicide	H_2O	JA_1	JA_2	JA_3	Fungicide	H_2O	
		%					%					
1	0.05								31.09	38.49	30.43	100
2	0.15								100.00			100
3	0.20							63.27		36.73		100
4	0.25	17.89	14.41	33.30			34.40					100
5	0.30				42.04	14.83	21.94		10.12	11.07		100
6	0.45	43.56	33.29	23.15								100
7	0.55				100.00							100
8	0.76	33.71			38.09			28.20				100
9	0.90	57.00			43.00							100
No. of bands		4	2	2	4	1	2	2	3	3	1	

3. Specific DNA bands (mostly two) are concomitant with JA treatments and the fungicide in the infected plants. Thus, in treatment with 5 or 10 mM JA, or the fungicide (in the infected plants), three unique DNA sequences were shown (222 bp with OPA-1, & 272 bp with OPA-2). On the other hand, also three DNA fragments were specific with 10 and 20 mM JA and the fungicide, in the infected plants (739bp with OPA-1, 396bp with OPA-2, & 350 bp by OPA-4). A similar trend, but to a lower extent, was also observed in the non-infected plants. This conclusion may reveal further that the fungicide effects more or less mimicked the effects displayed by JA (particularly 10mM), especially in the infected plants.

Peroxidase and esterase isozymes. The results presented in Fig. 3 and Table II indicates that the different concentrations of JA (5, 10 & 20 mM) and the fungicide (Rhizolex-T) enhanced the induction of three peroxidase isozymes in the fungus-infected plants. In absence of the fungal infection, the extracts of the control and the differently treated plants exhibited only two peroxidase isozymes. A similar result was also shown in the extract of the control (H_2O) plants non-infected with the fungus. The increase in peroxidase isozymes, in response to JA and the fungicide, might be a consequence of amplification of the jasmonate signal transduction pathway in consequence to exogenous application of either JA or the fungicide. Supplementation of JA is expected to enhance endogenous JA levels via autoinduction (Schaller, 2001), and as discussed above, the fungicide might be assumed to act through the JA cascade. In this connection, there is evidence that peroxidase isozymes can be induced by jasmonate (Curtis *et al*., 1997), wounding (Bernards *et al*., 1999), and pathogenesis (Lamb & Dixon, 1997). In such cases, they are included within the synthesis of lignin (Whetten *et al*., 1998) and mediation of cross linking of polysaccharides or extensions of the cell wall (Browleader *et al*., 2000; Takeda *et al*., 2003).

Table II also shows that JA at 10 and 20 mM as well as the fungicide induced a marked enhancement of peroxidase concentration in the infected plants. In the non-infected plants, a similar trend was observed with respect to treatments with the highest concentration of JA (20mM) and the fungicide. This result might be interpreted on the bases that peroxidases contribute in the consumption of H_2O_2 in both cross-linking reactions with pectic-associated phenols and in oxidative cleavage of these phenols (Kauss *et al*., 1993; Kim *et al*., 1998). Meanwhile, production of reactive oxygen species (ROS) in plants, particularly H_2O_2, is well documented as a key component in plant defensive responses (Lamb & Dixon, 1997). Furthermore**,** recent studies provide evidence that ROS function downstream of JA to amplify wound and systemin-induced responses (Li *et al*., 2001).

Fig. 4 and Table III show the changes in esterase isozymes, in response to JA and fungicide treatments in the

Fig. 3. Electrophoretic pattern of Peroxidase isozyme bands of faba bean plants (30-day-old). Lanes 1-5, represent plants infected with Fusarium wilt [1 (5 mM), 2 (10 mM), 3 (20 mM) jasomic acid, 4(fungicide), 5 (control H_2O)]. Lanes 6-10, represent non-infected plants [6 (5 mM), 7 (10 mM), 8 (20 mM) jasmonic acid, 9 (fungicide), 10 (control H_2O)]

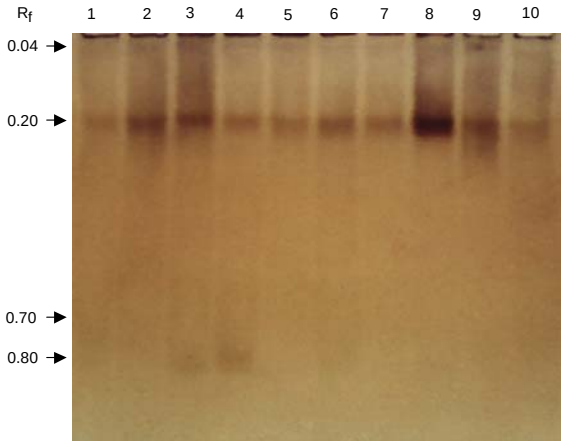
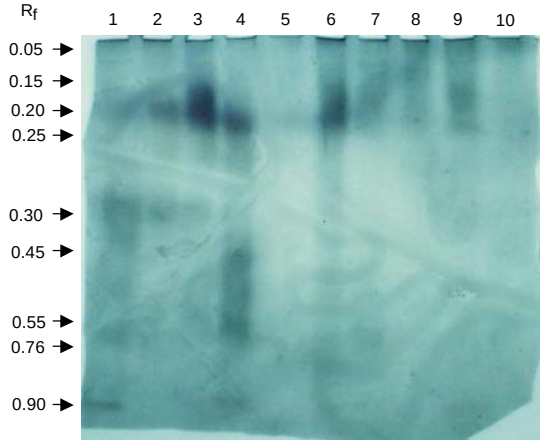


Fig. 4. Electrophoretic pattern of Esterase isozyme bands of faba bean plants (30-day-old). Lanes 1-5, represent plants infected with Fusarium wilt [1 (5 mM), 2 (10 mM), 3 (20 mM) jasomic acid, 4 (fungicide), 5 (control H_2O)]. Lanes 6-10, represent non-infectedplants [6 (5 mM), 7 (10 mM), 8 (20 mM) jasmonic acid, 9 (fungicide), 10 (control H_2O)]



infected and non-infected plants. These results indicate a higher number, than the control, of esterase isozymes in the infected plants as a result of treatments with 5, 10 & 20 mM JA or the fungicide. In this respect, the largest number of isozymes was shown in treatment with 5 mM JA or the fungicide. In the non-infected plants, the highest number of esterase isozymes was detected in treatments with 20 mM JA and the fungicide.

A marked increase in esterase concentration was also obtained, as a result of treatments with different concentrations (5, 10 & 20 mM) of JA and the fungicide, as compared with corresponding control (Table VI). The changes in the concentrations of esterase in the non-infected plants showed approximately similar trends. The interference of esterases in pathogenesis, might be via JA-related processes, due to esterase potential in hydrolyzing methyl JA, methyl esters of ABA, and fatty acids (Stuhlfelder *et al*., 2002).

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