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Full Length Research Paper

Biochemical screening of chocolate spot disease on faba bean caused by *Botrytis fabae*

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In this work, three faba bean cultivars were studied. The cultivar Giza 429 was the most susceptible cultivar to chocolate spot disease, followed by Giza 40, which was moderately susceptible; cultivar Giza 461 was found to be resistant. Four fungal species associated with diseased leaves were isolated from naturally infected samples and identified as *Botrytis fabae*, *Alternaria alternata* and *Stemphylium* sp. The most frequently isolated fungus was *B. fabae*, with an average isolation frequency of 51.03%. Of the *B. fabae* isolates, six were pathogenic in Giza 429 and Giza 40, and the symptoms of chocolate spot appeared three days after inoculation: *B. fabae* isolate No. 1 was the most virulent, whereas isolate No. 6 was the least virulent. The effect of *B. fabae* infection in the two varieties caused biochemical changes in the content of phenolics, oxidative enzymes, phytoalexins and free amino acids. HPLC analysis showed that the resistant cultivar Giza 461 demonstrated significantly higher amounts of oxidative enzymes, free, conjugated and total phenols and phytoalexins (wyerone acid, wyerone and wyerone epoxide) compared with the highly susceptible cultivar Giza 429. The results of this study may improve our understanding of the biochemical basis of resistance to *B. fabae* in the faba bean.

Key words: Faba bean, *Botrytis fabae*, chocolate spot disease, phenolics, oxidative enzymes and phytoalexins, fractionation of amino acids.

INTRODUCTION

The faba bean (*Vicia faba* L.) is considered as one of the most profitable field crops in Egypt. The plant is grown mainly for its green pods and dried seeds, which are rich in a protein (18.5 to 37.8%) that can substitute for animal protein in humans, as well as other compounds (El-Sayed et al., 1982). However, the faba bean is susceptible to attack by many diseases, such as chocolate spot disease caused mainly by *Botrytis fabae* (Metwally et al., 2010), and diseases are responsible for considerable losses in seed yield (Morsy, 2000; Daboor, 2001; Abo-bakr, 2002; Mazen, 2004; Eisa et al., 2006). Peroxidases oxidize phenols to quinines, which are toxic to pathogens (Bowles, 1990). Peroxidases participate in a broad range of physiological processes, such as the formation of lignin and suberin, the cross-linking of cell wall components, and phytoalexin synthesis. Peroxidase also functions in the metabolism of reactive oxygen species (ROS) and reactive nitrogen species (RNS), thus activating the hypersensitive response (HR), a form of programmed cell death at the infection site that is associated with limiting pathogen development (Almagro et al., 2009).

Polyphenoloxidases participate in the oxidation of aromatic substrates and dihydroxyphenolic compounds in the presence of oxygen in host tissues, producing quinones that are toxic to pathogens (Alfred, 2006). As major phytoalexins, wyerone and wyerone derivatives accumulate in infected tissues, leading to the inhibition of fungal growth (Buzi et al., 2003). Rapid wyerone acid accumulation was observed in the leaves of resistant cultivars and reached levels greater than twice that in susceptible cultivars (Nawar and Kuti, 2003). Free amino acids have been investigated in plants with chocolate spot, and the infection of faba bean with *B. fabae* led to changes in the balance of free amino acids (Medhat,

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2007).

The aim of this study was to evaluate the effects of *B. fabae* infection on oxidative enzyme activity and phenolic compound, phytoalexin and amino acid levels in three faba bean cultivars.

MATERIALS AND METHODS

Isolation, purification and identification of fungi from diseased faba bean leaves

Samples of naturally infected faba bean (*Vicia faba* L.) leaves were collected from three different areas: Nubaria (El-Beheira), Mansoura (El-Dakhilia), and El-Hamoul (Kafr-El Sheikh). Infected leaflets with symptoms of chocolate spot disease were cut into small pieces, each with a single lesion. The infected tissues were sterilized by soaking in 2% sodium hypochlorite for 2 to 3 min, rinsed with distilled water, dried on sterilized filter paper and then placed on PDA (Potato Dextrose Agar) media. Four pieces were placed onto each culture plate, and the plates were incubated at 20°C for 7 days. Pure isolates were obtained using single-spore or hyphal-tip techniques. The causative organisms were identified in collaboration with the Department of Mycology, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt.

Pathogenicity test of *Botrytis fabae*

The *B. fabae* pathogens collected from the three different regions were grown on FBLA medium (50 g faba bean, 30 g sucrose, 20 g sodium chloride and 20 g agar in one liter of distilled water, autoclaved for 15 min and poured into sterilized Petri dishes) to obtain a large quantity of spores (Leach and Moore, 1966). The plates were inoculated with discs (5 mm in diameter) of the six isolates (three isolates from El-Beheira, two isolates from El-Hamoul and one isolate from Mansoura) and incubated for 12 days at 20°C, with a photoperiod of 12 h light/12 h dark (five replicates for each isolate). After incubation, 10-ml aliquots of sterilized water were added to the cultures, and a fine brush was used to fragment the spores and suspend them in the water. A concentration of $2.5 \times l0^5$ conidia/ml was prepared for each isolated pathogen. Seeds from three faba bean cultivars, Giza 461, Giza 40 and Giza 429, were sown in pots (20 cm in diameter), with five seeds per pot (5 replicates for each cultivar). Forty-five days after sowing, the grown plants were sprayed with the spore suspension of each fungal isolate and covered with polyethylene bags for 24 h to maintain a high relative humidity (Abou-Zeid, 1985). Plants sprayed with distilled water were used as a control. The inoculated and uninoculated plants were maintained for 48 h at 20°C under greenhouse conditions. The infection type (disease severity) and severity of leaf damage (area) after 48 h were assessed using a scale (0 to 9) that depended on the extent of the lesions, as described by Abou-Zeid (1985).

$$\text{Disease severity \%} = \frac{n \times v}{9N} \times 100$$

where, n = number of plants in each category; v = numerical values of the symptom category; N = total number of plants; 9 = maximum numerical value of each symptom category.

Biochemical studies of plants with chocolate spot

Healthy and *B. fabae*-inoculated Giza 429 and Giza 461 plants were assessed for changes in oxidative enzyme (peroxidase and polyphenoloxidase) activities, phenolic compound levels, phytoalexin compound levels and free amino acid composition at 0, 6, 12, 18, 24, 30, 36, 40 and 48 h after inoculation.

Peroxidase and polyphenoloxidase activity

The leaf enzyme extract was prepared as recommended by Maxwell and Bateman (1967). The leaf tissues were ground with 0.1 M sodium phosphate buffer at pH 7.1 (2 ml buffer/g of fresh leaf tissue) in a mortar. The ground tissues were filtered through four layers of cheesecloth, and the filtrates were centrifuged at 3000 rpm for 20 min at 6°C. The supernatant was then subjected to enzyme assays. Peroxidase activity was estimated as described by Allam and Hollis (1972), and the polyphenoloxidase activity was measured using the colorimetric method described by Maxwell and Bateman (1967).

Phenolic compounds

The phenolic compound content was determined calorimetrically using Folin reagent as described by Snell and Snell (1953). 10 g of dried leaves were cut into small portions and immediately immersed into 95% boiling ethanol for 10 min. The samples were then extracted with 75% ethanol for 10 to 12 h in a Soxhlet unit, and the obtained ethanol extracts were filtered and evaporated to near dryness in a rotary evaporator at 60°C. The dried residue was re-dissolved in 6 ml of 50% isopropyl alcohol and used to determine the phenolic compound content. The phenolic compound content was determined calorimetrically using Folin reagent as described by Snell and Snell (1953).

Measurement of phytoalexins

The phytoalexin content was determined according to the method described by Christin (1990). 1 g of fresh tissue was homogenized with 40% methanol and agitated on a shaker for 2 h. The extract was filtered through Whatman No. 1 filter paper, and the solvent was evaporated in vacuo. The dry residue was dissolved in 1 ml of methanol: 0.1 M macetate buffer (pH 2) (1:1 v/v) and stored in vials. HPLC was used to detect the phytoalexins extracted from the plant tissue. The extract was filtered through a 0.45 µm microfilter, and the compounds were separated using a C18-SAX column (250 × 4.6 mm) with an HPLC (model HP 1050) equipped with a UV detector. The mobile phase consisted of methanol: 0.1 M acetate buffer (pH 2) (1:1 v/v). The detection was performed at 254 nm, and the total run time for the separation was approximately 25 min at a flow rate of 1 ml/min.

Fractionation of amino acids

The free amino acids were extracted according to the method proposed by Shad et al. (2002), as follows: 2 g of each sample were soaked separately in 75% ethanol (100 ml); after 24 h, the sample was ground and filtered. The residue was washed with 5 ml of 75% ethanol, and the volume was brought up to 100 ml. Selected amino acids were measured using an HPLC system (HP1050) with a UV detector at 254 nm. The separation was accomplished with an ODS C18 (5 µm 4 × 250 mm) column. The mobile phase consisted of 32% (acetonitrile/tetrahydrofuran, 90/10 v/v) and 64% (tetrahydrofuran/water, 5/95 v/v) with 0.3 ml acetic acid; the pH was adjusted to 5.15 with 1 M NaOH. The flow rate was 1.5 ml/min. The temperature of the column was 60°C, and the injection volume was 10 µl, as recommended by Christian (1990).

Table 1. The frequency of fungi isolated from the diseased leaves of faba bean plants collected from three governorates.

Governorate	Location	*B. fabae*	*A. alternata*	*Stemphylium* sp.
El Beheira	Nubaria	61.5	13.3	5.7
El Dakhilia	Mansoura	34.7	20.5	8.6
Kafr-El Sheikh	Sakha	46.9	11.6	6.3
Mean	-	51.03	13.46	6.86

Table 2. The pathogenicity of each isolate on two faba bean cultivars under greenhouse conditions.

Source	Isolate no.	Disease severity (%)	
		G. 429	G. 461
Nubaria	1	53.2	9.5
Nubaria	2	48.5	8.1
Nubaria	3	44.0	8.7
Sakha	4	40.0	5.5
Sakha	5	35.0	4.9
Mansoura	6	38.7	3.6
Mean	-	43.2	6.7

Statistical analysis

The statistical analysis was performed using the analysis of variance procedure described by Sendecor and Cochran (1980).

RESULTS

Isolation, purification and identification of fungi isolated from diseased faba bean leaves

Three fungal species associated with diseased leaves were isolated from naturally infected samples and identified as *B. fabae*, *Alternaria alternata* and *Stemphylium* sp. The prevalence of the fungi isolated from the diseased leaves of faba bean plants collected from different governorates is listed in Table 1. *B. fabae* was the most frequently isolated fungus, present in an average of 51.03% of samples from the three governorates. This species was isolated with the highest frequency from Nubaria, whereas the lowest frequency was detected in the plants from Mansoura. The most common symptoms on the leaves and stems were small (1 to 5 mm) circular to oblong, dark-red spots. As the disease progressed, the spots merged into large red areas on the leaves and stems. Next, large (8 to 12 mm) oblong to irregular dark-brown necrotic spots with concentric circles formed on the leaves, resulting in blighting and the death of the foliage (Koike, 1998).

Pathogenicity test of *B. fabae*

The results presented in Table 2 show that the six isolates were pathogenic to the three faba bean cultivars, with the symptoms of chocolate spot appearing at 3 days after inoculation. *B. fabae* isolate No. 1 from the faba bean plants grown in the Nubaria area was the most virulent, whereas isolate No. 6 from Mansoura caused the lowest disease severity.

Biochemical studies of plants showing chocolate spot disease symptoms

Peroxidase activity

The data presented in Table 3 indicate that the enzyme activities in the susceptible G. 429 cultivar increased when compared with the healthy plants. The highest value was 4.5 absorbance/min/g fresh weight at 6 h after inoculation, and the enzyme activity decreased at 48 h (1.0 absorbance/min/g fresh weight). The peroxidase activity of the resistant G. 461 cultivar at time zero was 6.0 absorbance/min/g fresh weight, increased to 10.7 at 6 h and decreased from 12 to 48 h.

Polyphenoloxidase activity

The data presented in Table 4 indicate that the polyphenoloxidase activity in the susceptible G. 429 cultivar increased in comparison to the healthy plants. Its value peaked at 4.4 absorbance/min/g fresh weight at 6 h and decreased at 48 h (1.2 absorbance/min/g fresh weight). The highest activity of polyphenoloxidase in the resistant G. 461 cultivar was 5.8 absorbance/min/g fresh weight at 12 h, and the polyphenoloxidase activity decreased thereafter. In general, the polyphenoloxidase activity in cv. G. 461 was higher than that in cv. G. 429.

Phenolic compounds

The free phenols were measured in the leaves of inoculated and uninoculated cultivars G. 429 and G. 461 at 6, 12, 18, 24, 30, 36, 42 and 48 h after inoculation with *B. fabae* under greenhouse conditions. The data presented in Table 5 reveal that the free phenols in the infected leaves were higher than those in the healthy plants. The free, conjugated and total phenols in the G.

Table 3. The peroxidase activity (absorbance/min/g fresh weight) of two different faba bean cultivars inoculated with *B. fabae* at different time points (h).

Time after inoculation (h)	Peroxidase activity (absorbance/min/g fresh weight)	
	G. 429	G. 461
0	1.9^{ab}*	6.0^{bc}
6	4.5^{a}	10.7^{ab}
12	4.3^{a}	10.0^{a}
18	4.0^{ab}	9.6^{ab}
24	2.9^{ab}	7.9^{ab}
30	2.8^{ab}	7.5^{ab}
36	2.0^{ab}	6.4^{ab}
42	1.3^{b}	5.2^{cd}
48	1.0^{ab}	4.5^{d}

Each values is the mean of 3 replicates; *, Level of significance of the standard deviations at $p < 0.05$. , data followed by the same letter are not significant at $P \leqslant 0.05$, but followed by different letters are significant at $P \leqslant 0.05$).

Table 4. The polyphenoloxidase activity (absorbance/minute/g fresh weight) of two different faba bean cultivars inoculated with *B. fabae* at different time points (h).

Time after inoculation (h)	Polyphenoloxidase activity (absorbance/min/g fresh weight)	
	G. 429	G. 461
0	1.8^{bc}*	4.2^{a}
6	4.4^{ab}	5.3^{a}
12	4.2^{a}	5.8^{a}
18	4.1^{ab}	5.4^{a}
24	2.8^{bc}	5.1^{a}
30	2.7^{bc}	4.7^{a}
36	2.0^{c}	4.5^{a}
42	1.4^{c}	3.9^{a}
48	1.2^{c}	3.7^{a}

Each values is the mean of 3 replicates. * Level of significance of the standard deviations at $p < 0.05$. , data followed by the same letter are not significant at $P \leqslant 0.05$, but followed by different letters are significant at $P \leqslant 0.05$).

Table 5. The phenolic content (mg catechol/5 g fresh weight) of two different faba bean cultivars inoculated with *B. fabae* at different time points (h).

Time after inoculation (h)	Phenols content (mg catechol/5 g fresh weight)					
	G. 429			G. 461		
	Free phenols	Conj. phenols	Total phenols	Free phenols	Conj. phenols	Total phenols
0	1.9^{de}*	8.3^{de}	10.2^{de}	2.3^{bc}	8.9^{bc}	11.2^{a}
6	2.2^{cd}	9.4^{cd}	11.6^{cd}	2.7^{ab}	9.6^{ab}	12.3^{a}
12	2.6^{bc}	10.1^{bc}	12.7^{bc}	3.4^{ab}	10.9^{a}	14.3^{a}
18	2.8^{bc}	11.5^{ab}	14.3^{ab}	3.6^{a}	11.5^{bc}	15.1^{a}
24	3.1^{bc}	11.7^{ab}	14.8^{ab}	3.9^{a}	12.3^{a}	16.2^{a}
30	3.5^{ab}	12.1^{a}	15.6^{a}	3.5^{a}	11.9^{bc}	15.4^{a}
36	3.7^{ab}	12.4^{a}	16.1^{bc}	3.1^{cd}	11.4^{bc}	14.5^{a}
42	3.4^{de}	11.9^{ab}	15.3^{bc}	2.9^{de}	11.1^{cd}	14.0^{a}
48	3.2^{e}	11.6^{e}	14.8^{e}	2.5^{e}	10.1^{d}	12.6^{a}

Conj, Conjugated; each values is the mean of 3 replicates ; * Level of significance of the standard deviations at $p < 0.05$. , data followed by the same letter are not significant at $P \leqslant 0.05$, but followed by different letters are significant at $P \leqslant 0.05$).

Table 6. The wyerone derivative levels (mg catechol/5 g fresh weight) of two different faba bean cultivars inoculated with *B. fabae* at different time points (h).

Time after inoculation (h)	Wyerone derivative content (mg/1 g fresh weight)					
	G. 429			G. 461		
	Wyerone acid	Wyerone	Wyerone epoxy	Wyerone acid	Wyerone	Wyerone epoxy
0	0.0^{a}*	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
6	17^{g}	14^{f}	15^{d}	17^{g}	15^{f}	14^{f}
12	24^{b}	18^{de}	21^{c}	31^{f}	32^{e}	23^{e}
18	39^{ef}	23^{c}	29^{b}	43^{e}	41^{d}	35^{c}
24	56^{c}	32^{b}	37^{a}	81^{a}	58^{b}	47^{a}
30	67^{b}	46^{a}	20^{c}	72^{b}	64^{a}	41^{b}
36	33^{a}	21^{cd}	18^{e}	63^{c}	57^{b}	36^{b}
42	28^{d}	16^{ef}	12^{f}	52^{d}	49^{c}	29^{d}
48	21^{fg}	13^{f}	9^{f}	41^{e}	40^{d}	22^{e}

The values are the mean of 3 replicates; * Level of significance of the standard deviations at $p < 0.05$. , data followed by the same letter are not significant at $P \leqslant 0.05$, but followed by different letters are significant at $P \leqslant 0.05$).

429 cultivar increased at 36 h, reaching 3.7, 12.4 and 16.1 mg catechol/5 g fresh weight, respectively, and decreased thereafter. In G. 461, the free, conjugated and total phenols increased from 6 to 24 h, with the highest values being 3.9, 12.3 and 16.2 mg catechol/5 g fresh weight, respectively, and decreased thereafter.

Phytoalexins

The phytoalexin (wyerone acid, wyerone, and wyerone epoxide) content in the leaves of inoculated and uninoculated G. 429 and G. 461 plants were examined at 6, 12, 18, 24, 30, 36, 42 and 48 h after inoculation with *B. fabae* under greenhouse conditions. As shown in Table 6, no wyerone acid or wyerone content was observed in the control samples. The amount of wyerone acid and wyerone in the susceptible G. 429 cultivar increased from 6 to 30 h, after which the wyerone acid and wyerone content decreased to 21 and 13 µg/g fresh weight at 48 h. The amount of wyerone acid in the resistant G. 461 cultivar increased from 6 to 24 h, and the wyerone acid content then decreased to 41 µg/g fresh weight at 48 h. The wyerone content increased from 6 to 30 h after inoculation and then decreased. In addition, the highest amount of wyerone epoxy in G. 429 or G. 461 was 37 and 47 µg/g fresh weight, respectively, at 24 h and then decreased to 9 and 22 µg/g fresh weight at 48 h.

Fractionation of amino acids

The free amino acids in the leaves of the inoculated and uninoculated G. 429 and G. 461 plants were measured at 6, 12, 18, 24, 30, 36, 42 and 48 h after inoculation with *B. fabae* under greenhouse conditions.

Cultivar G. 429: Infection increased the amino acid content in the leaves of the G. 429 cultivar, as shown in Table 7. Proline, cysteine, arginine, methionine and histidine increased from 6 to 24 h after inoculation, with the highest values recorded at 24 h (58, 42, 57, 65 and 42 ppm/5 g fresh weight, respectively). The maximum increases in threonine, phenylalanine, glutamic acid and tyrosine were recorded at 30 h (32, 51, 45 and 50 ppm/5 g fresh weight, respectively), and the levels of these amino acids decreased thereafter.

Cultivar G. 461: The results presented in Table 8 indicate that all amino acid levels were higher in inoculated G. 461 plants compared with uninoculated plants. The amino acids proline, cysteine, methionine, phenylalanine and tyrosine increased from 6 to 36 h after inoculation, reaching maximum values of 66, 52, 64, 70 and 71 ppm/5 g fresh weight at 36 h. Arginine, threonine and glutamic acid increased from 6 to 24 h, reaching maximum values of 55, 52 and 48 ppm/5 g fresh weight. Histidine increased from 6 to 30 h and reached a maximum value of 47 ppm/5 g fresh weight.

DISCUSSION

Three fungal species associated with diseased leaves were isolated from naturally infected samples. The isolated fungi were identified as *B. fabae*, *A. alternata* and *Stemphylium* sp. The most frequently isolated fungus was *B. fabae*, found in an average of 51.03% of the samples from the three governorates, a result that was consistent with previous reports (Abo-bakr, 2002; Mazen, 2004; El-Gammal, 2005; Eisa et al., 2006).

The six isolates were pathogenic against the three cultivars tested. The *B. fabae* No. 1 isolate from the faba

Table 7. Free amino content (ppm/ 5 g fresh weight) of the faba bean cultivar G461 inoculated with *B. fabae* at different time points (h).

Time after inoculation (h)	G. 429								
	Free amino acid content (ppm/5 g fresh weight)								
	A	B	C	D	E	F	G	H	I
0	42^{c}*	25^{d}	38^{d}	40^{d}	35^{ab}	25^{a}	41^{ab}	40^{a}	45^{ab}
6	45^{b}	27^{b}	43^{cd}	49^{cd}	33^{bc}	22^{a}	42^{ab}	38^{a}	48^{ab}
12	49^{b}	33^{ab}	48^{cd}	56^{ab}	35^{bc}	25^{a}	45^{ab}	41^{a}	50^{ab}
18	55^{ab}	38^{ab}	52^{cd}	63^{ab}	39^{ab}	27^{a}	46^{ab}	42^{a}	51^{ab}
24	48^{a}	42^{a}	57^{a}	65^{a}	42^{a}	31^{a}	48^{ab}	46^{a}	54^{a}
30	53^{ab}	39^{ab}	55^{a}	60^{ab}	40^{a}	32^{a}	51^{ab}	45^{a}	50^{ab}
36	51^{ab}	35^{ab}	53^{bc}	56^{bc}	37^{a}	30^{a}	47^{ab}	43^{a}	46^{ab}
42	47^{b}	3^{ab}	48^{bc}	51^{cd}	32^{bc}	26^{a}	44^{ab}	40^{a}	41^{ab}
48	45^{b}	29^{b}	42^{cd}	48^{d}	28^{c}	23^{a}	39^{b}	39^{a}	37^{c}

A = Mean of proline; B = mean of cysteine; C = mean of arginine; D = mean of methionine; E = mean of histidine; F = mean of threonine; G = mean of phenylalanine; H = mean of glutamic acid; I = mean of tyrosine. the values are the mean of 3 replicates; * Level of significance of the standard deviations at $p < 0.05$. , data followed by the same letter are not significant at $P \leqslant 0.05$, but followed by different letters are significant at $P \leqslant 0.05$).

Table 8. Free amino content (ppm/ 5 g fresh weight) of the faba bean cultivar G461 inoculated with *B. fabae* at different time points (h).

Time after inoculation (h)	G. 461								
	Free amino content (ppm/5 g fresh weight)								
	A	B	C	D	E	F	G	H	I
0	50^{a}*	34^{b}	41^{a}	45^{bc}	32^{a}	32^{c}	49^{ab}	38^{a}	52^{ab}
6	56^{a}	37^{ab}	45^{a}	47^{c}	35^{a}	37^{bc}	52^{b}	41^{a}	56^{ab}
12	58^{a}	39^{ab}	48^{a}	51^{ab}	37^{a}	43^{bc}	56^{ab}	43^{a}	59^{ab}
18	61^{a}	42^{ab}	51^{a}	56^{ab}	40^{a}	49^{ab}	63^{ab}	46^{a}	62^{a}
24	62^{a}	46^{ab}	5^{a}	58^{ab}	43^{a}	52^{ab}	67^{a}	50^{a}	66^{a}
30	65^{a}	49^{ab}	53^{a}	61^{a}	47^{a}	48^{ab}	68^{ab}	48^{a}	67^{a}
36	66^{a}	52^{ab}	50^{a}	64^{bc}	45^{a}	45^{ab}	70^{ab}	56^{a}	71^{a}
42	63^{a}	48^{ab}	47^{a}	62^{bc}	41^{a}	41^{a}	67^{b}	42^{a}	68^{ab}
48	61^{a}	45^{ab}	43^{a}	59^{ab}	38^{a}	37^{a}	65^{ab}	41^{a}	65^{b}

A = Mean of proline; B = mean of cysteine; C = mean of arginine; D = mean of methionine; E = mean of histidine; F = mean of threonine; G = mean of phenylalanine; H = mean of glutamic acid; I = mean of tyrosine. each values is the mean of 3 replicates; * Level of significance of the standard deviations at $p < 0.05$. , data followed by the same letter are not significant at $P \leqslant 0.05$, but followed by different letters are significant at $P \leqslant 0.05$).

bean plants grown in the Nubaria area was the most virulent, whereas isolate No. 6 from Mansoura caused the least severe disease.

Peroxidase has different functions in different defense mechanisms, e.g., the generation of ROS, suberin synthesis, lignin synthesis and the oxidation of phenols to derivatives that are toxic to pathogens.

ROS are toxic intermediates that result from the successive single-electron steps in the reduction of molecular O_2. The predominant species detected in plant-pathogen interactions are superoxide (O_2^-), hydrogen peroxide ($H_2O_2^-$) and the hydroxy radical (OH^-). The oxidative burst is correlated with hypersensitive response in a number of plant-pathogen interactions, and therefore may be an important element of disease resistance.

Peroxidase is thought to be involved in the polymerization of phenolic monomers to generate the aromatic matrix of suberin. Suberization is a developmentally regulated process but can also be induced site-specifically when a diffusion barrier must be constructed for defense purposes.

The final stage of lignin biosynthesis involves the

polymerization of cinnamyl alcohols to phenoxy radicals that couple nonenzymatically to produce oligomers of increasing sizes (Bowles, 1990). Among the chemicals whose expression is induced by pathogens, the simple phenolic compounds are the most inhibitory. In addition, polyphenoloxidase and peroxidase are activated by pathogens, resulting in the oxidation of phenolics to form quinones, which are effective inhibitors of phytopathogens. In addition, a large number of toxic phytoalexins can be derived from phenolic compounds (Orcutt and Nilsen, 2000).

Different studies have shown that biochemical resistance through the accumulation of various phenolic compounds and phytoalexins and the activation of peroxidases, polyphenoloxidases and key enzymes in the phenylpropanoid and isoflavonoid pathways may play a crucial role in the biological control of and resistance to pathogenic attack in plants. The defense strategy of plants consists of two stages. The first stage is assumed to involve the rapid accumulation of phenols at the infection site, which function to slow the growth rate of the pathogen and to allow for the activation of "secondary" strategies that will more thoroughly restrict the pathogen. The secondary responses involve the activation of specific defense mechanisms, such as the synthesis of molecules related to pathogen stress (Mohamed et al, 2007).

Wyerone acid and wyerone (an acetylenic furanoid keto-ester) are fungitoxic compounds. Wyerone acid inhibits *Botrytis cinerea* but is much less effective against *B. fabae*, which degrades this phytoalexin. The peroxidase activity in uninfected and infected leaves and the wyerone acid biosynthesis in infected faba bean plants were successfully used to ascertain the resistance and susceptibility of four faba bean cultivars to *B. fabae*, that is, these compounds are preliminary markers for the resistance of faba bean to the chocolate spot disease caused by *B. fabae* (Nawar and Kuti, 2003).

Faba bean leaves accumulate furanoacetylenic phytoalexins in response to the infection by *B. fabae*, and according to the results of the HPLC analysis, wyerone and wyerone acid are the two main phytoalexins that accumulate in leaves in response to the infection. Because wyerone is highly fungitoxic, it seems reasonable to conclude that the accumulation of phytoalexins restricts the growth of hyphae during resistance response. However, *B. fabae* is able to metabolize and detoxify these inhibitory compounds in the leaf tissues, thereby preventing their accumulation around the invading hyphae (Tarrad et al., 1993).

The levels of free amino acids in faba bean leaves during chocolate spot infection have been investigated (Habib, 1990; Ahmed, 2001); the levels increase after infection and the magnitude of increase is more pronounced in the susceptible cultivar (Mohamed et al., 1995).

These results could be interpreted to indicate that the fungus interferes with amino acid metabolism, thus furnishing adequate nutrients for its own benefit in the susceptible host. The increase in the free amino acid content in the infected tissues may also be attributed to the decomposition of host proteins or decreased protein synthesis. Although certain differences were found in the concentrations of some amino acids at different stages of host development, no individual amino acids could be defined as characteristic of a highly susceptible or resistant healthy cultivar (Medhat, 2007).

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