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Control of Chocolate Spot Disease by Non Traditional Methods on Faba Bean Plants

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Some chemical inducers, *i.e.* ascorbic, citric, salicylic acids and calcium chloride (as a nutrient salt) were evaluated to control faba bean chocolate spot disease. All tested organic acids significantly reduced the *in vitro* mycelial growth of the pathogenic fungus *(Botrytis fabae* Sard.) and complete inhibition of growth was recorded when 2500 ppm concentration was applied. Salicylic acid was the most effective one followed by ascorbic acid. Plant treatment with organic acids and calcium chloride under greenhouse and/or field conditions led to significant effect on controlling the disease and increasing phenols content as well as the activity of oxidative enzymes in the two tested varieties, *i.e.* Giza3 and Giza716, compared to control treatment. Giza3 was more susceptible than Giza716 and less in chemical components.

Keywords: Calcium chloride, chocolate spot disease, faba bean, organic acids, oxidative enzymes and phenols content.

Faba bean (*Vicia faba* L.) is a major food crop for Egyptian consumers. Faba bean is very important as a human food, animal feed and its beneficial effects in improving the soil fertility (Anonymous, 2005). This strategic crop is suffering from many destructive diseases. Chocolate spot disease of faba bean caused by *Botrytis fabae* Sard. was the most important limiting factor which causes great annual losses and sometimes complete crop failures (Koike, 1998). It is considered as the most destructive disease affecting faba bean crop and causing serious damage to the plant and consequently decreases the seed yield production especially in North Egypt (Abou-Zeid and Hassanein, 2000). Chocolate spot disease caused mainly by *B. fabae* Sard. which can spread and sporulate from lesions (Harrison, 1988). This destructive disease often causes considerable yield losses, especially after periods of high humidity and low temperature (Gaunt, 1983 and Mahmoud, 1996). Chemical inducers seem to predispose the original defence mechanisms in plants against diseases or produce some new compounds supporting it. Resistance does not exist against all diseases and the breeding for resistant plants take many years. Moreover, acceptance of genetically engineered resistance is still a sensitive issue in the European Union. The wide spread use of fungicides to control plant diseases has led to an increase of health hazards due to their phytotoxic residual and pollution effects, therefore using some other means of disease control instead of agrochemical is strongly encourage. Induced resistance is a promising modern approach with a broad spectrum in plant disease control. It could be induced in plants by applying chemical elicitors (Hassan *et al.*, 2007). Chemical inducers used to predispose the defence mechanisms in plants against diseases. Achuo *et al.* (2004) and many others used different inducers like ascorbic, salicylic and citric acids compared with

calcium chloride (Mazen, 2004). In addition to their ability to control diseases, field application of chemical elicitors of induced resistance, it is also important to study their effects on faba bean plants. Investigations of mechanisms of control the diseases revealed that calcium chloride is responsible for formation of strong cell walls (Suzuki *et al.*, 2003 and Abd El-Moneim, 2005).

Treating faba bean with chemical inducers simultaneously observed chocolate spot disease resistance were examined under greenhouse and field conditions. On the other hand, induced resistance may also affect phenol content and increasing activity of oxidative enzymes such as peroxidase, polyphenoloxidase and phenylalanine ammonia lyase (PAL) (Hammerschmidt *et al.*, 2001).

The present investigation was designed to isolate and identify the casual organism of faba bean chocolate spot disease. Evaluation the effect of ascorbic, citric and salicylic acids as well as calcium chloride as chemical inducers of resistance against faba bean chocolate spot disease was also considered. The effect of these inducers on some associated biochemical changes (phenol content and oxidative enzymes) in treated plants was also determined.

Materials and Methods

Isolation, purification and identification of the casual organism of faba bean chocolate spot disease:

Different plant parts (leaves, flowers and pods) of faba bean varieties (Giza3 and Giza716), showing symptoms of chocolate spot disease, were collected from growing areas located in Gilvina Valley at Sharkiya Governorate. Collected plant parts were surface sterilized with 5% sodium hypochlorite solution for 1-2 min, then washed in sterilized water and dried between two layers of sterilized filter papers. The infected parts were placed on faba bean agar (FBA) medium (Last and Hamley, 1956) and incubated at room temperature (20-25^{o}C). Any developed fungus was carefully transferred to slants gliotoxin fermentation agar (GFA) medium (Brian and Hemming, 1945). Isolated fungi were purified using the single spore technique (Hansen, 1926). The emerged fungi were identified according to their cultural and morphological characteristics as described by Morgan (1971) and Munjal (1980) and Barnett and Hunter (2003).

Pathogenicity test:

Three different *Botrytis fabae* isolates were evaluated for their pathogenic capabilities, using the intact leaves technique, on two faba bean varieties (Giza716 and Giza3) under greenhouse conditions at Agric. Res. Centre, Giza, during 2010 growing season. Tested seeds of faba bean varieties were obtained from Field Crop Res. Inst., Agric. Res. Centre, Giza, Egypt. Plastic pots (25-cm-diam.) were filled with 2kg clay soil. Five seeds of the desired tested faba bean variety were planted in each pot. Three replicates were used for each particular treatment. Re-isolation of the chocolate spot pathogenic fungus was carried out to confirm the pathogenicity test. Five ml of sterilized tap water were added to the surface of pure colonies in Petri dishes, gently shaked in a round motion until spores were released into the water. Fungal suspension was adjusted using sterilized water to contain $8x10^4$ spores

using a haemocytometer. The prepared suspension of the isolated pathogenic fungus was used to spray the two tested varieties (Giza716 and Giza3) and the percentages of disease severity were calculated, compared to control treatment (sprayed with water only). Numbers of the necrotic lesions as well as the size of blighted area per leaf were calculated according to the scale reported by Hanounik (1986). Different plant disease severity levels were recorded and a plant disease index was prepared to illustrate the symptoms in each category as follows:

1= No spots or very small non sporulating flecks
3= Few spots covering less than 1%of leaf area and few or no spots on the stems.
5= Spots common on leaves covering> 1-4% of leaf area, little defoliation and some spots on the stems.
7= Very common spots on leaves covering >4-8% of leaf area, some defoliation and many spots on the stems.
9= Extensive spots on leaves and stems covering >8-10% of leaf area, many dead leaves and severe defoliation.

Disease severity (DS) percentage was calculated using the following equation:

$$DS\ (\%) = (Sum\ (n \times v))/9N \times 100$$

Whereas: n = Number of plants in every grade
v = Numerical grade
N= Total number of leaves examined
9 = Maximum disease grade

Effect of different organic acid concentrations:
A) On B. fabae linear growth:

This experiment was designed to study the effect of different concentrations (100, 250, 500 1000 and 2500 ppm) of the tested organic acids (ascorbic, citric and salicylic acids) on the linear growth of *B. fabae.* One gram of the tested organic acid was added to one litre distilled sterilized water to obtain 1000 ppm stock concentration. Different organic acids concentrations were prepared from the stock solution and added to GFM at different rates to obtain acids concentrations. Media contain different concentrations (100, 250, 500, 1000 and 2500 ppm) from the tested organic acids were poured in plates at the rate of 15ml/plate. GFM with different concentrations of the tested organic acids were inoculated separately with an equal disc (0.5 mm) of the pathogenic fungus. Mycelial growth reduction (%) was calculated when the mycelial growth in control plates covered all the medium surface using the following equation:

$$\text{Mycelial growth reduction (\%)} = 100 - [G2 - G1/\ G2] \times 100$$

Whereas: G1= growth of pathogenic fungus in treated plates.
G2= growth of pathogenic fungus in control plates.

B) Under greenhouse conditions:

Faba bean plants representing the two tested varieties (Giza716 and Giza3) were grown under greenhouse conditions. Plants were kept at 20ºC and high relative humidity (90%). Intact leaves were inoculated with the spore suspension of *B. fabae*.

Ascorbic, citric and salicylic acids were used at the rate of 2000 ppm in addition to calcium chloride (1:200 ml) by using distilled water. Three replicates were used for each particular treatment. The leaves of faba bean plants varieties were sprayed with the pathogenic fungus spore suspension only and water as control treatment. All treatments were sprayed with all tested factors mentioned 3 times at 15 days intervals, starting from the 30th day after sowing. All plants were covered with polyethylene bags for 24 h. to maintain high relative humidity, and then kept under greenhouse conditions. Five randomly plants were selected and tagged plants from each pot were used to determine of chocolate spot disease severity.

C) Under field conditions:

This experiment was carried out under field conditions in Gilvina Valley at Sharkiya Governorate during the two tested growing seasons (2010 and 2011).

A randomized complete block design with 4 replications was used in each season. The experimental plot consisted of 6 ridges, 60 cm apart and 5 m in length (18 m^2 total areas). One seed, in each hill were sown in 15 October 2010 and 2011 to obtain final plant population. Organic acids were used at the rate of 2000 ppm and calcium chloride as nutrient salt (1:200 ml/distilled water) were used starting from the 30th day after sowing three times at 15 days intervals. Leaves sprayed with water only were served as control. Ten plants from each plot were at random harvested 90 days after sowing to determine the phenolic content and oxidative enzymes (peroxidase, polyphenoloxidase and phenylalanine ammonia lyase).

The percentages of disease incidence and disease severity of chocolate spot was assessed 45 and 75 days after sowing. Fifteen randomly plants selected from each plot were harvested and used to determine chocolate spot severity assessment according to the scale mentioned before.

Samples extraction:

One g of each infected and/or healthy leaf pieces of each treatment was added to 10 ml ethanol 70%, put on water bath at 70°C for 72 h and left to dry and then 5ml isopropanol 50% were added to the dry film and kept in deep freezer for chemical determination.

Determination of total phenols:

Free, conjugated and total phenols were determined by mixing one ml of the sample extract with 0.25 ml HCL and boiled in a water bath for 10 minutes then left to cool. One ml of the reagent folin-denis and 6 ml Na_2Co_3, were added. The mixture was completed to final volume (10 ml) using distilled water. Colour optical density of the reacted mixture was measured on absorbance spectrophotometer (Model: Miltonroy Spectronic 601) at 520nm (Snell and Snell, 1953). Phenol content was determined as mg/g fresh weight/min.

Enzyme extraction:

One gram of leaves samples from each faba bean treatment, healthy or infected was crushed well in 2 ml sodium phosphate buffer 0.1 µ at pH 7.1. The homogenate was filtrated through Whattman (No.1) filter paper. The suspension was centrifuged at 6000 rpm at 4^{o}C for 20 min and stored at 18^{o}C until use. One tenth extracted

sample was added to 0.5 ml sodium phosphate buffer 0.1 µ at pH 7.1, 0.1ml H_2O_2 1% and 0.3 ml pyrogallol 0.05 µ. Enzyme activity was calculated as mg/gm fresh weight.

Phosphate buffer preparation:

Eight grams of di-sodium hydrogen were dissolved in 250 ml of distilled water in a volumetric flask (solution A) and 3.9g sodium hydrogen phosphate were dissolved in 250 ml distilled water in another flask (Solution B). Solution A was added to solution B to have pH 7.1.

Peroxidase activity:

The mixture was completed to 3ml using distilled water and colour density was read in absorbance spectrophotometer (Model: Miltonroy Spectronic 601) at 425nm every 30 second for 10 reads (Kochba *et al*., 1977).

Polyphenoloxidase activity:

One tenth of the extracted sample was added to 0.5 ml sodium phosphate buffer 0.1 ml at pH 7and 0.5 ml catechol 0.001 N. The mixture was completed to 3ml using distilled water and colour density was read in spectrophotometer (Model: Miltonroy Spectronic 601) at 495nm every 30 second for 10 reads (Lisker *et al*., 1983).

Phenylalanine ammonia lyase (PAL) assay:

The enzyme preparation was obtained from acetone powders of faba bean leaves. 0.12g was blended for 1 min in 100 m acetone. The homogenate was filtered through Whattman No.1 filter paper, and then the residue was blended again using acetone. This step was repeated three times. The acetone powder was then air-dried at room temperature for 3 h. and stored at -18^{o}C until use. For the PAL assay, 500 mg of the dry acetone powder were added to 10 ml of cold 0.1 µ borate buffer, pH 8.8, and stirred for 1 h at 4^{o}C. The suspension was centrifuged twice (6000 rpm) at 4^{o}C and the clear suspension was dialyzed for 48 h at 4^{o}C in 0.2 µ borate buffers, pH 8.8 according to the method described by Lisker *et al.* (1983).

Statistical analysis:

Obtained Results were subjected to computer statistically analyzed according to the procedures of Snedecor and Cochran (1980). Means of all treatments were compared by the least significant difference LSD value at 5% level of probability.

Results and Discussion

Isolation and identification of the causal pathogens:

The casual organism was isolated from different diseased faba bean parts, *i.e.* leaves, flowers and pods, collected from Gilvina Valley, Sharkiya Governorate. Three fungal isolates, found to be the most frequently ones, were purified and identified as *Botrytis fabae* Sard.

Pathogenicity test:

Tested *B. fabae* isolates were found to be pathogenic, to different degrees, to the tested faba bean varieties (Table 1). Results clear that *B. fabae* isolate (No.1) from leaves was the most pathogenic one (37.4%) on Giza3, while isolate (No.3) from pods was the most effective (34.3%) on Giza716. Meanwhile, isolate (No.2) from

flowers was the least pathogenic one on the two tested faba bean varieties (Giza716 and Giza3). The pathogenic capabilities of the tested isolates in their population showed differences between potential hosts and host-parasitic reaction. Differences between varieties in their susceptibility might be due to the differences in their genetic makeup and their effect on some morphological characters and chemical components of the plants, as well as the environmental conditions that might affect host pathogen relationships and play a role in varietal susceptibility (Walker, 1975).

Table 1. Pathogenicity tests of three *B. fabae* isolates on two faba bean varieties

Tested isolate	Disease severity (%)	
	(Giza3)	(Giza716)
Isolate (No.1) from leaves	37.4	31.0
Isolate (No.2) from flowers	23.6	29.1
Isolate (No.3) from pods	33.9	34.3
Mean	31.6	31.5
LSD at 0.05	4.4	5.5

Effect of different concentrations of organic acids:

1- On the linear growth of B. fabae:

Different concentrations of organic acids were added to the medium to determine their effect on *B. fabae* linear growth. Results in Table (2) show that applying any concentration of the tested organic acids caused reduction in the pathogen mycelial growth compared to the control treatment.

Results also indicate that there is a positive correlation between concentrations and percentage of reduction in mycelial growth. It is also clear that tested organic acids at the same concentration varied in their effect against the pathogen. Moreover, the three tested organic acids completely inhibited the mycelial growth of *B. fabae* isolate No.1 when applied at 2500ppm. Salicylic acid was the most effective one followed by ascorbic acid. These findings are in harmony with those reported by Coquoz *et al.* (2007).

Table 2. Effect of different organic acids concentrations on the reduction percentage of mycelial growth on the chocolate spot pathogen

Organic acid concentration (ppm)	Reduction (%) in mycelial growth of *B. fabae* isolate No.1		
	Ascorbic acid	Citric acid	Salicylic acid
100	15.7	7.8	11.7
250	41.2	28.0	35.5
500	73.1	61.4	84.9
1000	90.2	83.9	93.2
2500	100.0	100.0	100.0
Control	0.0	0.0	0.0
Mean	53.4	45.8	54.2
LSD at 0.05	18.7	12.4	5.9

2- Under greenhouse conditions:

Results in Table (3) show the effect of some chemicals applied to induce resistance against chocolate spot pathogen. All tested organic acids as well as calcium chloride decreased the disease severity of chocolate spot, to different degrees, in comparison to control treatment. Results also showed that different treatments varied in their effect on controlling the disease. Variety Giza3 was more susceptible than var. Giza716. These results could be due to the differences in the chemical and morphological structure of leaves. These results are in harmony with those reported by Harrison (1988). Clear significant differences were recorded when disease severity values were compared to control. Results also reveal that salicylic acid was the most effective organic acid which decreased chocolate spot severity on var. Giza3 (51.1%) and calcium chloride on var. Giza716 (56.9%). The effect of organic acids as chemical inducers might be due to their role in induction of disease resistance (Coquoz *et al.*, 2007). Wickeramaarchchi *et al*. (2003) stated that calcium chloride play an important role against chocolate spot pathogen by increasing the thickness of the leaves cell wall and cuticle layer, so it protect the plant from invasion the fungal pathogen. Ryals and Ward (1994) mentioned that all plants have the ability to defend themselves against pathogenic infection through a wide variety of mechanisms that can be local or systemic and constitutive or inducible. This systemic acquired resistance (SAR) is a pathogen inducible defence mechanism that depended on salicylic acid (SA) and is associated with a system expression of a subset of defence genes and the acidic of pathogenesis-related proteins (PR) (Ward *et al.*, 1991). Inducers are used to initiate defence reactions as they denote something that can activate the plant's defensive mechanisms. The role of oxidative enzymes might be explained as oxidation of free phenols which accumulate according to the fungal growth. These newly synthesized polyphenols and their oxidative products might limit the fungal activity in the resistant tissue. It is also worthy to mentioned that the infected tissues might led to increase the activities of the oxidative enzymes within the infected tissue even for those of the resistant ones.

Table 3. Effect of some chemical inducers on the reduction percentage of chocolate spot disease severity on two faba bean varieties under greenhouse conditions

Chemical inducer	Reduction (%) in disease severity of *B. fabae* isolate No.1	
	Faba bean varieties	
	Giza3	Giza716
Ascorbic acid 2000 ppm.	50.0	22.7
Citric acid 2000 ppm.	44.9	36.2
Salicylic acid 2000 ppm.	51.1	44.9
Calcium chloride 1:200 ml.	50.9	56.9
Control	0.0	0.0
Mean	39.4	32.1
LSD at 0.05	2.7	8.2

Results in Table (4) clearly show that all tested chemical inducers increased the enzyme activities under artificial inoculation by *B. fabae* (No. 1) on faba bean plants compared to the control. Results indicate that enzyme activity was higher in the resistant varieties than in the susceptible one (Tarrod *et al.,* 1993).

Table 4. Effect of chemical inducers on oxidative enzymes in two faba bean varieties under greenhouse conditions

Chemical inducer	Chemical components as mg/g fresh weight/min in varieties					
	Giza3			Giza716		
	Peroxidase	Polyphenol oxidase	Phenyl alanine ammonia lyase	Peroxidase	Polyphenol oxidase	Phenyl alanine ammonia lyase
Ascorbic acid 2000 ppm.	0.72	0.11	1.50	0.66	0.07	1.50
Citric acid 2000ppm.	1.87	0.08	1.70	0.76	0.13	1.90
Salicylic acid 2000 ppm.	0.97	0.09	1.75	2.78	0.12	2.10
Calcium chloride 1:200 ml.	0.70	0.15	2.06	1.60	0.22	2.10
Control	0.55	0.03	0.13	0.61	0.06	0.18
Mean	0.96	0.09	0.14	1.28	0.12	1.56

Peroxidase, polyphenoloxidase and phenylalanine ammonia lyase activity was increased in var. Giza716 than in var. Giza3, after treating with chemical inducers, when compared with control treatment. The variability of varietal reactions between faba bean varieties might be attributed to the plant growth stage (Harrison, 1988). Citric acid was the most inducer that increased peroxidase activity to (1.87) in var. Giza3 and salicylic acid (2.78) in var. Giza716. Application of chemical inducers resulted in changes in peroxidase isozymes in both healthy and infected plants. Some treatments introduced new isozymes and/or increments in concentration of some isozymes, especially after infection with the pathogen (Vance *et al*., 1980 and Fry, 1982). Calcium chloride was the most effective one when increased polyphenoloxidase activity up to 0.15 and 0.22 in Giza3 and Giza716, respectively. These findings may be due to the responsibility of calcium chloride in the formation of strong cell walls (Suzuki *et al.,* 2003 and Abd El-Moneim, 2005). Polyphenoloxidase induced metabolization of these phenolic compounds into toxic forms (Chranowski *et al*., 2003). Calcium chloride was the most effective treatment that increased phenylalanine ammonia lyase (PAL) activity up to 2.06 in var. Giza3 and salicylic acid showed the highest value (2.11) in var. Giza716. More activity of PAL due to the effect of chemical inducers might have prevented fungal invasion and thus the activity was recorded at higher level. It is also found that polyphenoloxidase and peroxidase are the main enzymes that responsible for quality loss due to phenolic degradation.

Results in Table (5) show that calcium chloride was the most effective one in increasing total, free and conjugated phenols on var. Giza 3, while salicylic acid was the most effective one in case of var. Giza 716. High activities of tested host components could be considered as antioxidant mechanism for protecting plants detrimental effects of pectinase on the plant cell walls. The close relationship between the rate of faba bean cell wall breakdown and the rate of cell injury supports the fact that the cell wall breakdown is responsible for cell death (Basham and Bateman, 1975).

Table 5. Effect of chemical inducers on phenol components in two faba bean varieties under greenhouse conditions

Chemical inducer	Phenol components as mg/g fresh weight/min in varieties					
	Giza3			Giza716		
	Total phenols	Conjugated phenols	Free phenols	Total phenols	Conjugated phenols	Free phenols
Ascorbic acid 2000ppm.	4.47	1.66	2.81	3.91	1.79	2.11
Citric acid 2000ppm.	3.37	0.65	3.04	3.71	1.63	2.08
Salicylic acid 2000ppm.	3.69	1.56	2.13	5.19	2.51	2.68
Calcium chloride 1:200 ml.	4.72	1.64	3.08	5.17	2.66	2.51
Control	1.71	0.33	1.06	2.40	1.21	1.19
Mean	3.59	1.17	2.43	3.06	1.96	2. 11

3- Under field conditions:

Results in Table (6) show that all tested organic acids and calcium chloride caused significant reduction in disease incidence and disease severity on the two tested faba bean varieties, compared to the control treatment. Concerning var. Giza3, salicylic acid was the most effective organic acid when reduced disease incidence to 1.93% and disease severity to 18.29%, followed by citric acid, being 2.08% and 16.85%, respectively, in season 2010. On the other hand, no significant differences were found among the values (%) in season 2011. Ascorbic acid and calcium chloride recorded the same percentage (being 8.33%) of disease incidence while; citric acid was the most effective organic acid that reduced disease severity to 17.03%. The effect of these chemicals might be due to their direct effect or role in inducing disease resistance. This fact suggested that salicylic acid binding by protein caused inhibition of its catalase activity and induction of defence response. Salicylic acid essential for the development of SAR could possibly play a role in local resistance. The reduction of disease incidence and severity was increased to 4.17% and 15.35%, respectively, by applying calcium chloride on var. Giza716 in season 2010. Calcium chloride plays a role in increasing the thickness of the cell wall and cuticle layer, so it protects faba bean plants from invasion by the fungal pathogen (Mendez *et al.,* 1994 and Wickeramaarchchi *et al*., 2003). In season 2011,

Table 6. Effect of some chemical inducers on the percentage of disease incidence and severity against chocolate spot on two faba bean varieties under field conditions 2010/2011

Percentage of disease incidence and severity on varieties								
Chemical inducer	Giza3				Giza716			
	Season (2010)		Season (2011)		Season (2010)		Season (2011)	
	Disease incidence	Disease severity	Disease incidence	Disease severity	Disease incidence	Disease severity	Disease incidence	Disease severity
Ascorbic acid	4.86	24.93	8.33	21.24	11.11	24.16	19.43	26.79
Citric acid	2.08	16.85	11.11	17.03	5.56	28.07	20.14	24.21
Salicylic acid	1.93	18.29	10.42	22.49	5.56	22.86	11.11	26.77
Calcium chloride	3.47	22.75	8.33	23.74	4.17	15.53	13.83	26.77
Control	7.64	35.88	13.59	26.18	11.11	38.87	23.13	38.87
Mean	3.99	23.74	10.36	22.14	7.50	25.89	17.53	28.68
LSD at 0.05	1.96	7.76	4.62	7.53	2.77	7.39	5.02	8.03

salicylic acid was the most effective (11.11%) on disease incidence while; citric acid reduced the disease severity to 24.21%. This might be due to the fact that organic acids induced the resistance in the plant and their effect on the physiological procedures and the chemical reactions in the plant cell which led to enhancement the acquired resistance (Coquoz *et al.,* 1995). These might be attributed to the differential mode of action of these chemicals. The mode of action of chemical inducers for controlling plant diseases might include: (1) acting as second messengers in enhancing the host defence mechanism (Geetha and Shetty, 2002); (2) activating resistance by increasing the activity of peroxidase (POD), the synthesis of new POD isoform, or the accumulation of the phenolic compounds (Sarma *et al*., 2007); (3) activating resistance through inhibition of some antioxidant enzymes and catalases, there by leading to production of elevated amounts of H_2O_2 accumulation (Radwan *et al*., 2008) and (4) enhancing resistance by direct effects on multiplication, development and survival of pathogens or indirect effects on plant metabolism, with subsequent effects on the pathogen food supply (Khanam *et al*., 2005). As evident from the different mode of action of the chemical inducers, the varying efficiencies among these chemicals in protecting faba bean against chocolate spot disease have been observed under greenhouse and field conditions. Differences between districts in percentage of disease incidence and severity might be due to the differences in relative humidity, temperature, time of leaf witness, sowing varieties, time of sowing and the race of the pathogen (Cole *et al*., 1996).

Results in Table (7) indicate that calcium chloride caused the highest increase (0.680) in peroxidase activity in var. Giza3, while ascorbic acid caused the highest activity (0.634) in var. Giza716 during season 2010. Variations in peroxidase activities were noticed between the two varieties in season 2010. Meanwhile, during season (2011), ascorbic acid was the most effective one in Giza3 and Giza 716, being 0.764 and 0.769, respectively. Peroxidase also catalyzes the final polymerization step of lignin synthesis, and might therefore be directly associated with the increased ability of systemically protected tissue to be lignified and that

Table 7. Effect of some chemical inducers on the chemical components in two faba bean varieties during 2010 and 2011 growing seasons

Chemical inducer	Chemical components as mg/g fresh weight/min in varieties											
	Giza3						Giza716					
	Peroxidase		Polyphenol oxidase		Phenyl alanine ammonia lyase		Peroxidase		Polyphenol oxidase		Phenyl alanine ammonia lyase	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Ascorbic acid	0.65	0.76	0.14	0.05	2.01	1.35	0.63	0.77	0.13	0.17	1.67	2.01
Citric acid	0.48	0.65	0.12	0.04	1.99	1.52	0.51	0.73	0.14	0.17	1.79	1.66
Salicylic acid	0.60	0.59	0.12	0.13	2.01	2.38	0.62	0.67	0.13	0.17	1.75	1.76
Calcium chloride	0.68	0.58	0.16	0.11	2.10	1.65	0.52	0.75	0.19	0.24	2.09	1.57
Control	0.42	0.56	0.10	0.08	1.11	0.88	0.45	0.64	0.12	0.11	1.10	0.68
Mean	0.57	0.63	0.13	0.08	1.84	1.56	0.55	0.71	0.14	0.17	1.68	1.54

may restricted the penetration (Gross, 1979). The increase in polyphenol oxidase activity has an important function in secondary cell wall biosynthesis by polymerizing hydroxyl and methyl hydroxylcinnamic alcohols into lignin and forming rigid cross-links between cellulose, pectin hydroxylproline in glycoproteins (HPLGP) and lignin (Grisebach,1981). The role of oxidative enzymes such as peroxidase could be explained as an oxidation process of phenol compounds to oxidize products (quinones) which may be limiting the fungal growth. Another explanation was achieved by Tarrod *et al.* (1993) who reported that increase in peroxidase activity; enhances lignifications in response to chocolate spot infection which may restrict the fungal penetration. Peroxidase also produces free radicals and hydrogen peroxide which are toxic to many microorganisms (Pena and Kuc, 1992). Another supportive suggestion was brought by Nawar and Kuti (2003) who stated that an increase in peroxidase activity is considered as a preliminary indicator for resistance of broad beans to chocolate spot disease. These compounds act as barriers against pathogen invasion.

Calcium chloride was the most chemical inducer in season 2010, when increased polyphenoloxidase to 0.16 and 0.19 in Giza3 and Giza 716, respectively, when compared with control treatment. Meanwhile, in season 2011, salicylic acid recorded the highest increase (being 0.13) in var. Giza3 while, calcium chloride reached (0.24) in Giza716. Polyphenoloxidase values were increased in var. Giza 716 than those in var. Giza3 during the two tested seasons. Polyphenoloxidase is widespread enzyme found in plant cells and located in the chloroplast membranes. This enzyme is capable of dehydrogenating of O-diphenols to produce O-quinones. However, it indicates the highest activity toward hydroxylation of monophenols to diphenols. Oxidation of phenolic compounds in plant cells is responsible for initiating the browning reaction of the tissues and is identified as presence of the pathogenic factor. Moreover, Polyphenoloxidase induces metabolization of these phenolic compounds into toxic forms (Chranowski *et al*., 2003). Phenyl alanine ammonia-lyase (PAL) is an enzyme of the general phenylpropanoid metabolism and controls

a key branch point in the biosynthetic pathways of flavanoid phytoalexins which are antimicrobial compounds (Lamb and Dixon, 1997).When calcium chloride was used, the activity of PAL was increased and this was noticed in the two varieties during season 2010, being 2.10 and 2.09, respectively. On the other hand, salicylic acid was the most effective in Giza 3 variety while ascorbic acid was the best for Giza716 at season 2011, being 2.38 and 2.01, respectively. More activity of PAL, due to the effect of chemical inducers may prevent fungal invasion and thus the activity was recorded at higher levels. The tested chemical inducers may stimulate some defence mechanisms such as phenolic compounds and oxidative enzymes. In the present work, the activity of peroxidase and polyphenoloxidase and phenylalanine ammonia lyase enzymes was obviously higher in treated plants compared to the untreated ones except in some few treatments. The present results are in agreement with those recorded by Ibrahim (2006). Oxidative-reduction enzymes played an important role in induced resistance by the oxidation of phenols to oxidized products (Quinine) which limited the fungal activity (Hassan *et al.*, 2006).

Results in Table (8) indicate that all phenol contents, *i.e.* total, free and conjugated, were increased in var. Giza 716 than in var. Giza3 during the two excremental seasons. Calcium chloride was the most effective one in increasing total, free and conjugated phenols (being, 8.35, 5.45 and 2.90, respectively), in the two tested varieties during season 2010. Calcium chloride is responsible for formation strong cell walls (Suzuki *et al.*, 2003). Citric acid recorded the highest increase of phenol contents in var. Giza3, being 7.84, 5.05 and 2.79, respectively, while, salicylic acid was the best for Giza 716, being 9.48, 7.16 and 2.68, respectively, during season 2011. The chemical effect of organic acids as antioxidant plays a clear role in improving plant physiology and metabolism. Phenols have been recorded to offer plant resistance against diseases. Also, plants containing high amount of polyphenols show high resistance degrees to several plant diseases (Malick and Singh, 1980).

Table 8. Effect of some chemical inducers on the phenol components in two faba bean varieties during 2010 and 2011 growing seasons

Chemical inducer	Phenol components as mg/g fresh weight/min in varieties											
	Giza3 (var.1)						Giza716 (var.2)					
	Total Phenols		Conjugated phenols		Free phenols		Total phenols		Conjugated phenols		Free Phenols	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Ascorbic acid	6.45	6.21	3.59	3.49	2.86	2.71	7.32	9.21	5.42	7.08	1.90	2.13
Citric acid	6.31	7.84	4.55	5.05	1.75	2.79	8.32	8.32	5.64	6.49	2.68	1.83
Salicylic acid	7.86	7.22	5.15	5.12	2.71	2.10	6.22	9.48	2.01	7.16	4.21	2.68
Calcium chloride	8.35	7.41	5.45	5.11	2.90	2.31	9.21	9.38	7.08	6.65	2.13	2.73
Control	5.73	5.77	4.31	3.13	1.42	2.64	5.78	6.22	2.80	4.21	2.98	2.01
Mean	2.82	6.89	4.61	4.38	2.23	2.51	7.37	8.52	4.59	6.32	2.78	2.27

It is well known that high levels of phenolic compounds such as quinone are highly toxic for plants and also inactivate the pectic enzymes secreted by the pathogen. Current promising results would lead to use chemical inducers as protective control means, since the curative mechanism of them still not fully investigated yet. No restrictions are needed to use such chemicals and no doubt, they are environmentally safer, economically cheaper and easy to use on the level of small farms with much less hazards comparing with ordinary fungicides. On the contrary, many points of study related to chemical inducers must be investigated before being commercially accepted and released.

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مقاومة مرض التبقع الشيكولاتي ببعض الطرق غير التقليديةفي نبا تات الفول

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تم تقييم رش نباتات الفول البلدى ببعض المستحثات الكيميائية (حامض الأسكوربيك ، حامض الستريك ،حامض السالسيلك) و كلوريد الكالسيوم (كملح مغذي) لمقاومة مرض التبقع الشيكولاتيبي في الفول البلدي. تم دراسة التأثير التضادي للأحماض العضوية وذلك تحت ظروف المعمل حيث إنخفض النمو الميسليومى للفطر الممرض بواسطة الأحماض العضوية انخفاضا معنويا وأدي الي أيقاف نمو الفطر تماما عند تركيز2500 جزء في المليون. وكان حامض السالسيلك أكثرهم تأثيرا يليه حامض الأسكوربيك. تم تطبيق كل المعاملات السابقة على مستوى الصوبة و الحقل ولوحظ أن إنخفاض نسبة وشدة الإصابة كان مصحوباً بزيادة تدريجية فى نشاط الفينولات والأنزيمات المؤكسدة في صنفي جيزة ٣ وجيزة ٧١٦ . وكان صنف جيزة ٣ أكثر قابلية للأصابة عن صنف جيزة ٧١٦ وأقلهم في التغيرات الكيماوية.