

Role of Reactive Oxygen Species in Defense Responses Against Chocolate Spot Disease in Resistant and Susceptible Faba Bean (*Vicia faba*) Cultivars

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ABSTRACT: Three infected faba bean (FB) plant cultivars (susceptible and resistant to *Botrytis fabae* Sard.) were pretreated with three tested biological inducers (*Trichoderma viride*, *Talaromyces stipitatus* and *Verticillium lecanii*). Disease index (DI) and disease severity (DS) were significantly reduced as a result of all treatments, Giza 40 showed less reduction compared with the infected and uninfected control. Later on activities of peroxidase (POD) and polyphenol oxidase (PPO), two infected bean cultivars were increased significantly compared with control. Treatment with the tested biological inducers resulted in significant increase in POD enzyme activity early at 24 hrs after inoculation of both susceptible (Giza 40) and resistant (Sakha2) cultivars with *B. fabae*, compared with the treated control. Moreover, *T. stipitatus* bioinducer recorded the highest POD activity values in all the tested cultivars. PPO enzyme activity significantly increased when pretreated resistant cv (Sakha2) with *T. stipitatus* and susceptible cv (Giza 40) with *V. lecanii*, compared with the other tested treatments. Increases in PPO activity were early observed in all treatments, except for *T. stipitatus*-Giza 40cv treatment. Increases and accumulation of reactive oxygen species (ROS), mainly superoxide ($O_2^{\cdot-}$) were detected at early stages (24 hrs) after inoculation due to pretreatment with *V. lecanii*, compared to untreated control of Giza 40 and treated control of Sakha2, giving the same results. At the meantime, it was found that pretreatment of Giza 40 and control with *T. stipitatus* significantly increased and accumulated levels of ($O_2^{\cdot-}$) at late stages (72 hrs) after inoculation. It was also noted that all treatments resulted in significant increase and accumulation of $O_2^{\cdot-}$, except for Giza 40 cv, which showed pronounced decrease in $O_2^{\cdot-}$ levels. In spite of the increase and accumulation of hydrogen peroxide (H_2O_2), detected in all treatments 72 hrs after inoculation, however, pretreated control of Giza 40 cv showed early increase and accumulation of H_2O_2 24 hrs after inoculation. Positive relationship between accumulated of H_2O_2 and the time elapsed after inoculation was observed in all the tested treatments.

Keywords: bio-agents inducers, faba bean, *Botrytis fabae*, *Trichoderma viride*, *Talaromyces stipitatus*, *Verticillium lecanii*, disease index (DI), disease severity (DS), peroxidase (POD), polyphenol oxidase (PPO), superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2).

INTRODUCTION

Faba bean (*Vicia faba* L.) is subjected to many abiotic and biotic stresses that seriously compromise the final yields (El-Hendawy *et al.*, 2010). Among the biotic stresses, the chocolate spot disease caused mainly by *Botrytis fabae* Sard, and to some extent *B. cinerea* Pers. is one of the most economically important diseases that damage the foliage, limit photosynthesis activity, and reduce FB production (Torres *et al.*, 2004). Chocolate spot is one of the most important diseases infecting FB in Egypt. In Morocco, Nile Delta or African regions, losses in yield due to chocolate spot disease can reach 60-80% among susceptible cultivars and up to 34% among tolerant cultivars (Bouhassan *et al.*, 2004, and Sahileet *et al.*, 2008). Chocolate spot, which is incited by the necrotrophic plant pathogen *Botrytis fabae* Sard., is one of the devastating diseases of FB (*Vicia faba* L.) production worldwide (Rhaïem *et al.*, 2002, and

Torres *et al.*, 2004), including Egypt (Abo-Hegazy *et al.*, 2012, Abou-Zeid *et al.*, 1990, Khalil and Harrison, 1981). The pathogen attacks all of the aboveground parts of the FB plant, thereby causing chocolate-colored lesions that may spread quickly around the infection site, killing the tissue above the lesion (Bouhassan *et al.*, 2004). In Egypt, yield losses exceed 20-25% (Khalil *et al.*, 1993) and may reach 100% under severe epidemic conditions (Bouhassan *et al.*, 2004, and Torres *et al.*, 2004). The deployment of resistant FB varieties is an efficient strategy for controlling the disease and promoting the development of sustainable agriculture (Bouhassan *et al.*, 2004, and Rhaïem *et al.*, 2002). However, only limited studies exist on the defense responses of the FB×*B. fabae* interaction (Sillero *et al.*, 2010, and El-Komy, 2014). The results of El-Komy *et al.* (2015) indicated that the chocolate spot pathogen induced lipid peroxidation and the production of reactive oxygen species, peroxidase (POD), and polyphenol oxidase (PPO) in leaf tissue during the FB×*B. fabae* interaction. The production of these defense compounds in leaves was not static but governed in time and extent by physiological maturity.

Plants defend themselves against fungal infection through the activation of complex defense responses (Dangl and Jones, 2001). One of the earliest these responses is the rapid generation of reactive oxygen species (ROS), which includes superoxide anion (O_2^-), hydroxyl radical (OH^\bullet) and hydrogen peroxide (H_2O_2) (Patykowski and Urbanek, 2003). There are numerous studies demonstrating the production of these free radicals in plants as an initial response to microorganisms, both pathogenic and non-pathogenic (Bolwellet *et al.*, 2002, Garcia-Limonet *et al.*, 2002, and Gill and Tuteja, 2010). ROS may have multiple effects on defense responses, including direct antimicrobial action, lignin biosynthesis, phytoalexin production and the triggering of systemic acquired resistance (Bradely *et al.*, 1992, Lamb and Dixon, 1997, Laloie *et al.*, 2004, and Peng and Kuć, 1992). Nevertheless, the excess ROS causes oxidative damage, leads to lipid peroxidation and damages macromolecules such as pigments, proteins, nucleic acids and lipids (Apel and Hirt, 2004). To limit oxidative damage, plants have a defense antioxidative system to modulate the cytotoxic effects of these free radicals, which include enzymes such as superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX) and catalase (CAT) (Mittler, 2002). However, the role of ROS in the defense mechanism against necrotrophic fungi (e.g., *Botrytis* spp.) remains controversial (Asselberghet *et al.*, 2007). Some studies have demonstrated a positive effect of ROS on plant resistance (Malolepsza and Urbanek, 2000, and Unger *et al.*, 2005). In contrast, other studies have demonstrated that the level of ROS accumulation in plant tissues was proportionally correlated with the aggressiveness of the necrotrophic pathogens (Govrin and Levine, 2000, Mayer *et al.*, 2001, and von Tiedemann, 1997).

The aim of this study was to identify reliable biological inducers pretreatments on FB resistance against chocolate spot. In this regard, the disease severity and the accumulation levels of POD and PPO enzymes as well as O_2^- , H_2O_2 , were investigated in, resistant Sakha 2 and susceptible Giza 40 FB cvs.

MATERIALS AND METHODS

Sources of cultivars

Three FB cultivars (Giza 40, Nubaria 1 and Sakha 2) were used throughout this investigation. These cultivars were obtained from Agricultural Research Center (ARC), Giza, Egypt, Nubaria Agriculture Research Centre, (El-Beheira) and Sakha Agriculture Research Centre (Kafr-El Sheikh).

Sources of fungal isolates

Botrytis fabae isolate (F3) were used throughout this study. Two biological inducer agents, including *T. viride* and *T. stipitatus* were obtained from the stock cultures from Laboratory of Plant Pathology, Agriculture Botany department at Agriculture Faculty (Saba-Basha), Alexandria University, whereas one fungal isolate, *V. lecanii* (Zimmerman) were obtained from Alaska, USA and EMCC Number: 919TM (Egypt microbial culture collection).

Experiments assay

FB seeds of Sakha 2 and Nubaria 1 resistant cvs and the susceptible cv Giza 40 were surface-sterilized with 1% sodium hypochlorite for 30 second and then rinsed in sterile distilled water and dried under a sterile air stream, and then were sown in plastic pots under greenhouse conditions.

The bio-based resistance inducer isolates *T. viride*, *T. stipitatus* and *V. lecanii* were cultivated on potato dextrose agar medium (PDA) at 25°C and maintained at 4 °C. Suspensions of bio-inducers was prepared in sterile distilled water from 7-days-old-culture on PDA (Rojo *et al.*, 2007). The fungal inoculum was harvested by flooding the culture with sterile distilled water (SDW) (10 ml), and then rubbing the culture surface with a sterile glass rod. The concentration of bio-inducers was adjusted to 1.0E07 spores/ml by dilution and direct counting using a haemocytometer (Emiko *et al.*, 2008, and Abd El-Rahman and Mohamed, 2014).

Forty days after sowing, the growing plants were sprayed until dropping with an individual spore suspension of the tested bio-inducers (*T. viride*, *T. stipitatus* and *V. lecanii*), at concentration 1.0E07 spores/ml on lower leaf surface (first and second leaves). Tween20 was added to spore suspension at concentration of 0.03%. Four days later, plants were sprayed with bio-inducers, with spore suspension of the tested pathogen at concentration (2.5E05 spores/ml). Control plants were sprayed with pathogen spore suspension only (without bio-inducers), whereas, plants sprayed with distilled water only were used as untreated non infected control. All cultivated pots were covered with polyethylene bags for 24 hrs under greenhouse conditions. Each treatment was represented by four pots served as a replicates. Disease severity was calculated using the devised scale of Bernier *et al.* (1993). Chocolate spot symptoms, appeared 7 days after inoculation, were scored as disease index (%) and disease severity (%) assessed according to Bernier *et al.* (1984) as follows:

$$\text{Disease severity (DS)\%} = \frac{\sum(\text{NPC} \times \text{CR})}{\text{NIP} \times \text{MSC}} \times 100$$

Where:

NPC = No. of plants in each class rate

CR = Class rate

NIP = No. of infected plants

MSC = Maximum severity class rate

$$\text{Disease index (DI)\%} = \frac{\text{number of infected plants}}{\text{total number of examined plants}} \times 100$$

Preparation of samples for physiological and biochemical experiments

After inoculation of plants with *B. fabae*, entire leaves (fourth and fifth) of each individual plant were collected and pooled at each sampling time (24, 48 and 72 hrs) and frozen in liquid nitrogen and kept at -80°C . These samples were then used for the preparation of total leaf homogenates required for enzymes assays.

Determination of enzymes activities

For determining enzyme activities of peroxidase (POD) and polyphenol oxidase (PPO) in plants, 0.5 g leaf material was homogenized at $0-4^{\circ}\text{C}$ in 3 ml of 50 mM TRIS buffer (pH 7.8), containing 1 mM EDTA- Na_2 and 7.5% polyvinylpyrrolidone (24, 48 and 72, hrs) after inoculation. The homogenates were centrifuged (12,000 rpm, 20 min, 4°C), and the total soluble enzyme activities were measured spectrophotometrically in the supernatant (Hafez, 2010). All measurements were carried out at 25°C , using the model UV-160A spectrophotometer (Shimadzu, Japan). This enzyme assays were tested three times. Each experiment was replicated three times.

Peroxidase (POD) activity

Peroxidase (POD) activity was directly determined of the crude enzyme extract according to a typical procedure proposed by Hammerschmidt *et al.* (1982). Changes in absorbance at 470 nm were recorded every 30 sec intervals for 3 min. Enzyme activity was expressed as increase in absorbance $\text{min}^{-1} \text{g}^{-1}$ fresh weight. Each experiment replicated three times.

Polyphenol oxidase (PPO) activity

Polyphenol oxidase (PPO) enzyme assays in plants was prepared as described above in peroxidase (POD) activity assay. Activity of polyphenol oxidase (PPO) was determined according to the method described by Malik and Singh (1980). Changes in the absorbance at 495 nm were recorded every 30 sec intervals for 3 min. Enzyme activity was expressed as increase in absorbance $\text{min}^{-1} \text{g}^{-1}$ fresh weight. Each experiment was replicated three times.

Determination of reactive oxygen species (ROS)

For determining of reactive oxygen species (ROS), all the fresh leaves (fourth and fifth leaves) were collected at different time intervals (24, 48 and 72 hrs) following inoculation. Superoxide $\text{O}_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) were visualized as a purple coloration of nitro blue tetrazolium (NBT) and a reddish-brown coloration of 3, 3-diaminobenzidine (DAB), respectively. It was measured at different time intervals (24, 48 and 72 hrs) following inoculation. Leaves of the susceptible (Giza 40), pretreated with *V. lecanii* and *T. stipitatus* were vacuum infiltrated with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 w/v %

NBT (Sigma–Aldrich, Steinheim, Germany) according to *Ádám et al.* (1989) or 0.1 w/v % DAB (Fluka, Buchs, Switzerland). NBT- and DAB-treated samples were incubated under daylight for 20 min and 2 hours, respectively and subsequently cleared in 0.15 w/v % trichloroacetic acid in ethanol: chloroform 4:1 v/v for 1 day (*Hückelhoven et al.*, 1999). Cleared samples were washed with water and placed in 50% glycerol prior to evaluation. Discoloration of leaf discs were photographed and resulted by NBT or DAB staining was quantified using a Chemilmager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA). This test was repeated three times. Uninfected leaves of susceptible cultivar Giza 40 were used as control (C1). In addition, non-pretreated infected FB leaves of both susceptible (Giza 40) and resistant (Sakha 2) cultivars were used also as control.

Statistical analysis

The experimental design was factorial in completely randomized design with four repetitions. The obtained data were subjected to analysis of variance according to Gomez and Gomez (1984). Treatment means were compared by Duncan's Multiple Range Test (Duncan, 1955). All statistical analysis was performed using analysis of variance technique by means of "MSTATC" computer software package.

RESULTS AND DISCUSSION

Effect of bio-inducer pretreatment on disease reduction

Comparing with the untreated infected and untreated non-infected control, considerable reduction in DI and DS percentages were recorded when bio-agent inducers used to predispose FB plants to confront and control chocolate spot disease caused by *B. fabae*, as illustrated on Table 1 and 2. *T. stipitatus* and *V. lecanii* were superior in their effects by recording the lowest DI values of chocolate spot disease (Table 1), while *T. viride*, was the less effective. Differences in DI % between Nubaria 1 and Sakha 2 cvs were insignificant; however, their DI% values were significantly lower, compared to untreated infected control (C2). Results of DI% of infected controls shown in Table (1) confirmed that both Sakha 2 and Nubaria 1 cvs were resistant against the disease, where DI% were 43.75 and 37.50%, respectively, whereas Giza 40 was highly susceptible (DI% was 81.25%).

The same trend of DS values was noticed in Table (2) where differences between Sakha 2 (3.33%) and Nubaria 1 (4.578%) cvs were insignificant. However, DI% values both of them were significantly lower than the susceptible Giza 40 (16.25%). Differences in DS% among the three tested bio-agent inducers treatments (*V. lecanii*, *T. stipitatus* & *T. viride*) were insignificant within each cultivar. Moreover, Differences in mean values of DS% among the tested bio-inducers were insignificant. Pretreatment with the tested bio-inducers before inoculation of the tested cvs with *B. fabae* resulted in sound significant reduction in DS% values, compared to untreated inoculated control (C2). In addition, the highest reductions in DS% in pretreated plants were detected in the resistant Sakha 2 and Nubaria 1 cvs. These findings were in harmony with results obtained by Haggag *et al.* (2006), who reported that *T. flavus* and *T. harzianum* reduced chocolate spot disease severity on FB. According to this study, lower disease severity values were recorded in plants pretreated with *T.*

flavus and *T. harzianum* bio-inducers (1.2-6.4% and 2.4-12.5%, respectively, compared to control (32.6%)) (Ermias *et al.*, 2013).

Table (1): Effect of treatment with the tested bioinducers on brown spot disease incidence (DI %), in different faba bean cultivars.

Treatments (T)	Disease Incidence (DI %)			
	Cultivars (C)			
	Giza 40	Sakha 2	Nubaria 1	Average
<i>T. viride</i>	62.50 ab	25.00 cde	12.50 de	33.33 B
<i>T. stipitatus</i>	31.25 cd	31.25 cd	18.75 cde	27.08 B
<i>V. lecanii</i>	43.75 bc	37.50 bcd	37.50 bcd	39.58 B
C2	81.25 a	43.75 bc	37.50 bcd	54.17 A
C1	0.00 e	0.00 e	0.00 e	0.00 C
Average	43.75 A	27.50 B	21.25 B	
LSD_{0.05}	(T)= 14.2		(C)= 11.01(T x C)= 24.61	

Where: C1= untreated non-inoculated control; C2= untreated inoculated control; Giza 40= susceptible cultivar; and Sakha2 and Nubaria 1= resistant cultivars; and *T. viride*, *T. stipitatus* and *V. lecanii*= Bio-agent inducers.

Table (2): Effect of treatment with the tested bioinducers on brown spot disease severity (DS %), in different faba bean cultivars.

Treatments (T)	Disease Severity DS%			
	Cultivars (C)			
	Giza 40	Sakha 2	Nubaria 1	Average
<i>T. viride</i>	9.028 c	2.777 d	1.390 d	4.398 B
<i>T. stipitatus</i>	5.555 cd	4.863 cd	2.085 d	4.168 B
<i>V. lecanii</i>	4.170 cd	4.170 cd	4.170 cd	4.170 B
C2	62.50 a	4.863 cd	15.24 b	27.54 A
C1	0.00 d	0.00d	0.00 d	0.00 C
Average	16.25 A	3.335 B	4.578 B	
LSD_{0.05}	(T) = 2.988		(C) = 2.315(T x C)= 5.176	

Where: C1= untreated non-inoculated control; C2= treated inoculated control; Giza 40= susceptible cultivar; and Sakha2 and Nubaria 1= resistant cultivars; and *T. viride*, *T. stipitatus* and *V. lecanii*= Bioagent inducers.

These observations on the effect of pretreatments with bio-inducers on DS% of both susceptible (Giza 40) and resistant (Sakha 2) cvs proved that various responses towered reduction of DI and DS%. Moreover, current findings revealed that *V. lecanii* and *T. stipitatus* were more efficient in reducing DI and DS than *T. viride*, similar results were obtained by El-Komy (2014) on the reactions of the two FB cultivars expressing different levels of resistance

against to *B. fabae*, Nubaria (resistant) and Giza 40 (susceptible) cultivars, using whole plant assays to avoid responses induced by wounding and not associated with the infection. For both cultivars, there was significant increase in disease severity over time. The progress of the disease symptoms evolved slowly on resistant cultivar but occurred at a much greater rate on the susceptible cultivar. However, disease severity was significantly lower for Nubaria cv than for Giza 40 cv, regardless of the time elapsed after inoculation. These results were in agreement with those of Abo-Hegazy *et al.* (2012), who confirmed the resistance of Nubaria cv and the susceptibility of Giza 40 cv. to infection by *B. fabae*. The lowest levels of disease severity recorded in the leaves of Nubaria 1 and Sakha 2 cvs suggest that resistance events that delay tissue colonization after fungal infection play a role in the FB x *B. fabae* interaction (Hanounik and Maliha, 1986, and Hanounik and Robertson, 1988).

Effect of pretreatment on oxidative enzymes activity: Peroxidase activity (POD)

Results recorded in Table 3 showed that peroxidase activity values significantly affected according to the tested cultivar, bio-inducer and time elapsed after inoculation. Pretreatment of faba bean plants with the tested bio-inducers before inoculation with *B. faba* resulted in insignificant changes in POD enzyme activities during the first day after inoculation (Table 3). Moreover, differences in POD activity values among all the tested treatments during the first 24 hours period after inoculation were insignificant.

After 48 hours of inoculation, sound significant differences in POD activities were detected in both resistant (Sakha 2) and susceptible (Giza 40) faba bean cultivars, pretreated with the tested *T. stipitatus* bio-inducer, compared with untreated non inoculated (C1) and inoculated (C2) control. Moreover, increase in POD activities was significantly higher in resistant Sakha 2 cv than susceptible Giza 40 cv (0.1997 and 0.1245, respectively). Pretreatment with the bio-inducer *V. lecanii* resulted in insignificant differences in POD activities throughout all the tested periods, compared with control. According to the mean values of POD activities of the three tested time periods, pretreatment with *T. stipitatus* significantly increased POD activities in both resistant and susceptible cvs (0.0573 and 0.0800, respectively); however, differences between the two bio-inducers were insignificant.

Therefore, according to data recorded in Table 3, it could be concluded that the bio-inducer *T. stipitatus* proved to be effective in increasing POD activities in both tested resistant and susceptible cvs during the second day after inoculation. Moreover, increase in POD activities induced by *T. stipitatus* two days after inoculation was higher in the resistant than the susceptible cv.

Our findings indicate the positive relationship between resistance of faba bean against chocolate leaf spots and peroxidase activity. These findings were in line with those reported by Ride (1983) and Tarrad *et al.* (1993), who found that increase in peroxidase activity enhance lignification in response to chocolate spot infection which may restrict the fungal penetration. Moreover, Nawar and Kuti (2003) concluded that peroxidase activity is considered as a preliminary indicator for resistance of broad beans to chocolate spot disease.

Table (3): Changes in peroxidase enzyme (POD) activities in resistant and susceptible faba bean cultivars pretreated with the tested bioagent inducers and inoculated with *B. fabae* at different periods following inoculation.

Cultivars (C)	Treatments (T)	Peroxidase activity (absorbance min ⁻¹ g ⁻¹ fresh weight)			
		Time after inoculation (P)			
		24 hrs	48 hrs	72 hrs	Average
		C x T x P- average			C x T- average
Giza40	C1	0.0052 c	0.0168 c	0.0173 c	0.0131 B
	C2	0.0147 c	0.0103 c	0.0061 c	0.0104 B
	<i>T. stipitatus</i>	0.0276 c	0.1245 b	0.0198 c	0.0573 A
	<i>V. lecanii</i>	0.0271 c	0.0207 c	0.0130 c	0.0203 B
Sakha2	C1	0.0102 c	0.0173 c	0.0111 c	0.0129 B
	C2	0.0138 c	0.0152 c	0.0041 c	0.011 B
	<i>T. stipitatus</i>	0.0086 c	0.1997 a	0.0316 c	0.08 A
	<i>V. lecanii</i>	0.0216 c	0.0078 c	0.0069 c	0.0121 B
LSD_{0.05}		0.0519			0.0299
		C x P – average			C- average
Giza40		0.0186 b	0.0431 a	0.0140 b	0.0253
Sakha2		0.0135 b	0.0600 a	0.0134 b	0.029
LSD_{0.05}		0.022			NS
		T x P – average			T- average
C1		0.0077 b	0.0171 b	0.0142 b	0.013 B
C2		0.0142 b	0.0128 b	0.0051 b	0.0107 B
<i>T. stipitatus</i>		0.0181 b	0.1621 a	0.0257 b	0.0687 A
<i>V. lecanii</i>		0.0244 b	0.0142 b	0.0099 b	0.0162 B
LSD_{0.05}		0.0367			0.021
P - average		0.0161 B	0.0515 A	0.0137 B	
LSD_{0.05}		0.0184			

Where: C1= untreated non-inoculated control; C2= untreated inoculated control; Giza 40= susceptible cultivar; and Sakha2 and Nubaria 1= resistant cultivars; and *T. viride*, *T. stipitatus* and *V. lecanii*= Bioagent inducers.

Data obtained throughout this investigation showed the positive role of bio-inducers, in particular *T. stipitatus* in decreasing disease severity and enhancement of peroxidase activity, and hence induction of resistance. The role of oxidative enzymes such as peroxidase could be explained as an oxidation process of phenol compounds to oxidized products (quinones) which may limit the fungal growth (Vance *et al.*, 1980, and Tarradet *et al.*, 1993), oxidation of polymerization of hydroxycinnamyl alcohols to yield lignin (Fry, 1982, and Cvikrová *et al.*, 2006), production of free radicals and hydrogen peroxide which are toxic to many microorganisms (Peng and Kuc, 1992), and makes the cell wall more mechanically rigid by cross-linking matrix polysaccharide and glycoprotein molecules and consequently inhibit cell wall degrading enzymes of the pathogen (El-Komy *et al.*, 2015).

Polyphenol oxidase (PPO) activity

Pretreatment of faba bean plants of resistant cv Sakha 2 with *T. stipitatus* and *V. lecanii* resulted in pronounced increase of PPO enzyme activities after 24 hrs of inoculation (0.0226 and 0.0087, respectively) (Table 4), compared to inoculated control (C2) (0.0060). Moreover, enzyme activity was much higher in *T. stipitatus* pretreatment than that of *V. lecanii*. Meanwhile, differences in enzyme activity between susceptible Giza 40 cv and infected control were insignificant during the first 24 hrs after inoculation. After the second day of inoculation, significant increase in PPO activities in susceptible cv, pretreated with *T. stipitatus* and *V. lecanii*, compared with control, however, increase was higher in *V. lecanii* treatment (0.0040 and 0.0275, respectively). On the other hand, *T. stipitatus* was more effective in increasing PPO activities (0.0050), whereas PPO activity in *V. lecanii* treatment was insignificant, compared to the other tested treatments.

After 72 hrs of inoculation, differences in PPO activity values between bio-inducer-pretreated Giza 40 plants and control were insignificant. However, pretreatment of resistant Sakha 2 plants with *T. stipitatus* significantly increased PPO activities, compared to control. These results suggest that the expression of phenoloxidizing enzymes can be associated with the resistance to *B. fabae*. Nawar and Kutti (2003) also reported that PO activity could be used as a biomarker to evaluate the resistance reactions in FB plants against chocolate spot disease.

Peroxidase and polyphenol oxidase catalyzes the oxidation of phenolic compounds to quinones (antimicrobial compounds) (El Komy *et al.*, 2015), involved in IAA biosynthesis. Endogenous reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) were stimulated significantly early after natural infection consequently, later on activities of catalase (CAT), peroxidase (POX) and poly phenol oxidase (PPO) were significantly increased compared with the control (Hafez *et al.*, 2014).

Effect of pretreatment on ROS production

Level of Hydrogen peroxide (H_2O_2)

The obtained data of Table 5 and Figure 1 showed that the activities of hydrogen peroxide (H_2O_2) varied significantly according to the tested bio-inducer and time elapsed after inoculation. Generally, according to the mean values of H_2O_2 of each time interval, it was evident that the lowest H_2O_2 level (15.58) was obtained directly after inoculation, increased by time, reaching maximum levels 72 after inoculation (31.01).

Table (4): Changes in polyphenol oxidase enzyme (PPO) activities in resistant and susceptible faba bean cultivars, pretreated with the tested bioagent inducers and inoculated with *B. fabae* at different periods following inoculation.

Cultivars (C)	Treatments (T)	Polyphenol oxidase activity (absorbance min ⁻¹ g ⁻¹ fresh weight)			
		Time after inoculation (p)			
		24 hrs	48 hrs	72 hrs	Average
		C x T x P-average			C x T- average
Giza40	C1	0.0087 bc	0.00193 c	0.0030 c	0.0045 B
	C2	0.0012 c	0.0013 c	0.0044 bc	0.0022 B
	<i>T. stipitatus</i>	0.0010 c	0.0040 bc	0.0020 c	0.0023 B
	<i>V. lecanii</i>	0.0025 c	0.0275 a	0.0010 c	0.0103 AB
Sakha2	C1	0.0030 c	0.0025 c	0.0025 c	0.0027 B
	C2	0.0060 bc	0.0016 c	0.0036 bc	0.00381 B
	<i>T. stipitatus</i>	0.0226 ab	0.0050 bc	0.0040 bc	0.0105 AB
	<i>V. lecanii</i>	0.0087 bc	0.0019 c	0.0030c	0.0045 B
LSD_{0.05}		0.00164			0.009
		C x P – average			C- average
Giza40		0.0069 b	0.0057 c	0.0053 c	0.0060
Sakha2		0.0085 a	0.0092 a	0.0028 d	0.0068
LSD_{0.05}		0.0009			NS
		T x P – average			T- average
	C1	0.0098 ab	0.0218 a	0.0065 b	0.0127 A
	C2	0.0058 b	0.0022 b	0.0027 b	0.0036 B
	<i>T. stipitatus</i>	0.0036 b	0.0013 b	0.0040 b	0.0029 B
	<i>V. lecanii</i>	0.0118 ab	0.0045 b	0.0030 b	0.0064 AB
LSD_{0.05}		0.0116			0.006
P - average		0.0077 A	0.0071 A	0.0041 b	
LSD_{0.05}		0.0018			

Where: C1= untreated non-inoculated control; C2= untreated inoculated control; Giza 40= susceptible cultivar; and Sakha2 and Nubaria 1= resistant cultivars; and *T. viride*, *T. stipitatus* and *V. lecanii*= Bioagent inducers.

Table (5): level of hydrogen peroxide (H₂O₂) in faba bean resistant and susceptible cultivars pretreated with the tested bio-agent inducers and inoculated with *B. fabae* at different periods following inoculation.

Treatments (T)	Level of hydrogen peroxide (H ₂ O ₂) Arbitrary units			
	Time after inoculation (P)			
	24 hrs	48 hrs	72 hrs	Average
C1	11.16 e	18.51 de	24.67 bcd	18.11 C
C2	24.24 bcd	24.45 bcd	39.21 a	29.30 A
C3	14.59 e	25.11 bcd	32.82 ab	24.17 B
<i>T. stipitatus</i>	14.32 e	20.04 cde	30.40 b	21.58 BC
<i>V. lecanii</i>	13.59 e	19.82 cde	27.97 bc	20.46 BC
Average	15.58 C	21.58 B	31.01 A	
LSD_{0.05}	T = 4.56	P= 3.53	T X P= 7.9	

Where: C1= untreated non-inoculated control of susceptible cv Giza 40; C2= untreated inoculated control of Giza 40; C3= untreated inoculated control of resistant cv Sakha 2, *T. stipitatus* and *V. lecanii*= bioagent inducers.

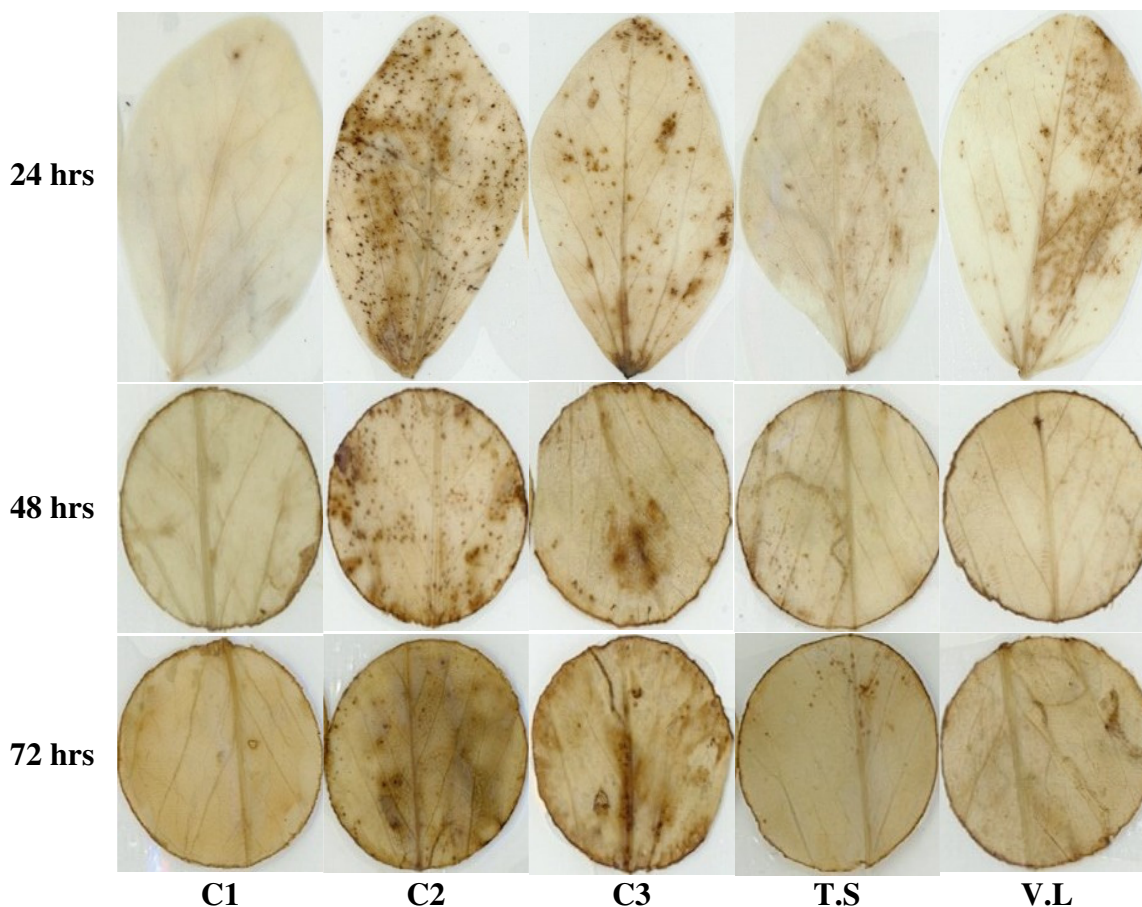


Figure (1): Brown discoloration (using DBA pigment) of hydrogen peroxide (H_2O_2) in both resistant and susceptible faba bean cultivars pre-treated with the tested bio-agent inducers and inoculated with *B. fabae* at different periods following inoculation.

Where: C1= untreated non-inoculated control of susceptible cv Giza 40; C2= untreated inoculated control of Giza 40; C3= untreated inoculated control of resistant cv Sakha 2, *T. stipitatus* and *V. lecanii*= bioagent inducers.

This was true in both untreated inoculated controls of both resistant and susceptible cvs (C1 and C2) and inoculated plants pretreated with the tested bio-inducers. Differences in H_2O_2 levels between the two tested bio-inducers *T. stipitatus* and *V. lecanii* during the three tested time periods were statistically insignificant. Hydrogen peroxide levels were higher in untreated inoculated control of susceptible Giza 40 cv (C2), compared to that of resistant Sakha 2 cv (C3), particularly in the first and third tested time periods (Table 6 and Figure1).

The obtained results indicated that the oxidative burst activities, which significantly varied among FB cultivars, are considered an essential process in defence mechanism. The higher levels of ROS accumulation were positively correlated with susceptibility of FB leaf tissues to infection with the necrotrophic fungus *B. fabae*, and significantly varied in intensity and induction timing according to FB resistance levels. These findings assured conclusions of many

published papers (von Tiedemann, 1997, Govrin and Levine, 2000, Mayer *et al.*, 2001 and Schouten *et al.*, 2002)

Moreover, pretreatment with the tested bio-inducers, *T. stipitatus* and *V. lecanii*, significantly increased the ROS level early, as a result, induction of the antioxidants occurred later, and accordingly the fungal growth was suppressed. These conclusions were in agreement with those reported by El-Komy (2014), who demonstrated that the accumulation of these defense responses was induced significantly in both cultivars upon infection with *B. fabae* compared with non-inoculated controls. He also found that resistant cultivar showed weaker necrotic symptom expression, less ROS accumulation, a lower rate of lipid peroxidation and higher activity of the enzymatic ROS scavenging system compared with susceptible cultivar.

In the present study, a significant increase in H₂O₂ generation in both FB cultivars was observed in response to *B. fabae* inoculation, compared with non-inoculated plants. Moreover, the greatest accumulation of H₂O₂ detected in the susceptible cultivar Giza 40, indicated that production of higher levels of ROS is positively correlated to the susceptibility of FB leaf tissues to infection with *B. fabae*. These findings confirmed the findings of many researchers (Garcia-Limones *et al.*, 2002, Mellersh *et al.*, 2002, and El-Komy, 2014). However, Govrin and Levine (2000) and Shi *et al.* (2011) showed that the inoculation of *B. cinerea* induced an oxidative burst in *Arabidopsis*, and the aggressiveness of this pathogen was directly dependent on the level of ROS accumulation. As a result of early stimulation of ROS, disease severity (%) and disease symptoms were suppressed. This perhaps attributed to the inhibiting or killing action of ROS to the fungal pathogen early after infection, therefore, there was no chance for the pathogen to grow or propagate (El-Zahaby *et al.*, 2004, Hafez, 2010, and Hafez *et al.*, 2014).

Level of superoxide (O₂⁻) using (NBT)

Data in Table 6 and Figure 2 showed significant increase in Levels of superoxide (O₂⁻) in untreated non inoculated faba bean plants of susceptible cv at the first and second day periods (38.33), however, significant drop was detected after the second and third day periods (14.76 and 27.55, respectively).

Pretreatment with *T. stipitatus* significantly reduced O₂⁻ level after 24 hrs of inoculation (14.10), compared to control, however, O₂⁻ levels gradually increased to reach maximum values (35.24) after the third day period. On the other hand, O₂⁻ level in *V. lecanii* treatment showed significant increase after the first day (23.13), sudden drop after the second day (17.84), and then reaching maximum levels 72 hrs after inoculation (26.44). According to Figure(1& 2), H₂O₂ and O₂⁻ levels were decreased due to the effect of pretreatment with both of the tested bio-inducers.

According to O₂⁻ level mean values of the three tested periods, O₂⁻ level in *T. stipitatus* treatment was higher than that of *V. lecanii* (24.01 and 22.47, respectively). Moreover, maximum levels of O₂⁻ were obtained 72 hrs after inoculation (Table 6 and Figure 2). The results of the present investigation indicated that the bio-inducers pretreatments increased the ROS level early within 72 hrs after inoculation, as a result, induction of the antioxidants was occurred later, and accordingly the fungal growth was suppressed.

These results were in great similarity with those obtained by Hafez *et al.* (2014), who concluded that the endogenous reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) were stimulated significantly early after natural infection consequently, later on activities of catalase (CAT), peroxidase (POX) and poly phenol oxidase (PPO) were increased significantly compared with the control. Mohamed *et al.* (2007) and Mohamed, *et al.* (2012) reported that peroxidase has different functions in different defense mechanisms.

Table (6): level of superoxide ($O_2^{\cdot-}$) by using NBT in faba bean resistant and susceptible cultivars pretreated with the tested bio-agent inducers and inoculated with *B. fabae* at different periods following inoculation.

Treatments (T)	Level of Superoxide ($O_2^{\cdot-}$) Arbitrary units			
	Time after inoculation (P)			
	24 hrs	48 hrs	72 hrs	Average
C1	38.33 a	27.55 cde	14.76 i	26.88 A
C2	16.52 hi	25.33 def	28.20 cd	23.35 BC
C3	25.77 cde	20.48 fgh	30.83 bc	25.69 AB
<i>T. stipitatus</i>	14.10 i	22.69 efg	35.24 ab	24.01 ABC
<i>V. lecanii</i>	23.13 def	17.84 ghi	26.44 cde	22.47 C
Average	23.57 B	22.78 B	27.09 A	
LSD_{0.05}	T = 2.752	p = 2.131	T X p = 4.766	

Where: C1= untreated non-inoculated control of susceptible cv Giza 40; C2= untreated inoculated control of Giza 40; C3= untreated inoculated control of resistant cv Sakha 2, *T. stipitatus* and *V. lecanii*= bioagent inducers.

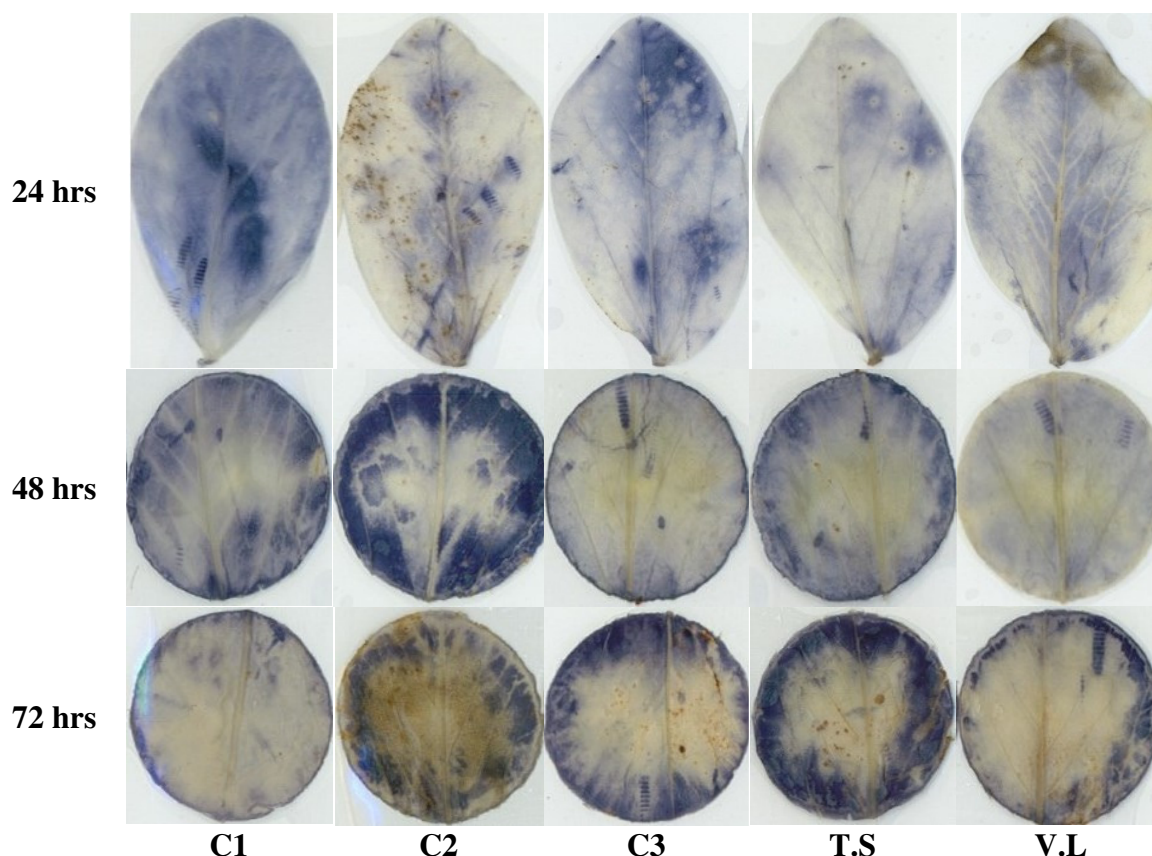


Figure (2): Purple discoloration (using NBT pigment) of superoxide ($O_2^{\cdot-}$) in both resistant and susceptible faba bean cultivars pre-treated with the tested bio-agent inducers and inoculated with *B. fabae* at different periods following inoculation.

Where: C1= untreated non-inoculated control of susceptible cv Giza 40; C2= untreated inoculated control of Giza 40; C3= untreated inoculated control of resistant cv Sakha 2, *T. stipitatus* and *V. lecanii*= bioagent inducers.

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الملخص العربي

دور أنواع الأوكسجين التفاعلي في الاستجابات الدفاعية في أصناف الفول المقاومة والحساسية للإصابة بمرض التبقع الشيكولاتي

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قسم النبات الزراعي - كلية الزراعة - جامعة كفر الشيخ - مصر

تمت الدراسة على ثلاثة أصناف من الفول أحدهما مقاوم والآخر حساس للإصابة بفطر بوتريتس فابي حيث تم معاملة النباتات مسبقاً بثلاث فطريات من مصادر الاستحثاث الحيوى هما فطر تريكوديرما فيريدي، تيارومييسيس ستيبييتيس و فيرتسيليوم ليكانياي. نسبة وشدة الإصابة بالمرض قد انخفضت معنوياً نتيجة تلك المعاملات، أظهر صنف جيزة ٤٠ إنخفاضاً أقل بالمقارنة بالكنترول الغير مصاب والكنترول المصاب الغير معاملة. تم زيادة أنشطة إنزيمات الأوكسيداز (POD) و البولى فينول أوكسيداز (PPO) متأخراً بدرجة معنوية فى كلا الصنفين المختبرين. إزدادت أنشطة انزيم الأوكسيداز POD نتيجة المعاملة المسبقة بالحاثات الحيوية مبكراً بعد ٢٤ ساعة من الإصابة بالمسبب المرضى فى كلا الصنفى الحساس (جيزة ٤٠) والمقاوم (سحا ٢). أظهرت النتائج أن الفطر تيارومييسيس

ستيبيتيس قد سجل أعلى كفاءة لانزيم البولى فينول أكسيديز (PPO) حيث ارتفع معنوياً عند المعاملة المسبقة بفطر تارومييسيس للصنف المقاوم (سحا ٢) وفطر فيرئيسيليوم ليكيناى للصنف الحساس (جيزة ٤٠) بالمقارنة بباقي المعاملات المختبرة. كما أن كفاءة انزيم البولى فينول أكسيديز (PPO) قد نشطت متأخراً فى كل المعاملات فيما عدا معاملة فطر تارومييسيس للصنف جيزة ٤٠. على الرغم من أن بعض المعاملات كانت قادرة على زيادة وتراكم مبكر لمستويات من (ROS) خاصة السوبر أكسيد (O_2^-) والذي أنتج مبكراً بعد ٢٤ ساعة والتي عوملت مسبقاً بفطر فيرئيسيليوم ليكيناى بالمقارنة بالكنترول الغير معامل لـ صنف جيزة ٤٠ والكنترول المعامل لـ صنف سحا ٢ والذي أعطى نفس النتيجة. بينما وجد أنه بعد مرور ٧٢ ساعة من العدوى فان المعاملة بفطر تارومييسيس والكنترول المعامل لـ صنف جيزة ٤٠ قادرين على زيادة وتراكم مستويات السوبر أكسيد متأخراً بعد العدوى. وتم أيضاً ملاحظة ان جميع المعاملات قد أدت الى زيادة وتراكم السوبر أكسيد بينما حدث إنخفاض مع صنف جيزة ٤٠. فى حين أن جميع المعاملات كانت قادرة على زيادة وتراكم مستويات الهيدروجين بيروكسيد (H_2O_2) متأخراً بعد العدوى بـ ٧٢ ساعة فيما عدا معاملة الكنترول المعامل لـ صنف جيزة ٤٠ والذي كان قادراً على زيادة وتراكم مبكر للهيدروجين بيروكسيد بعد ٢٤ ساعة من العدوى، وقد لوحظ أيضاً أن جميع المعاملات قد عملت على ارتفاع وتراكم الهيدروجين بيروكسيد (H_2O_2) مع زيادة الوقت بعد العدوى.

