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Full Length Article

Resistance/susceptibility of Faba Bean to *Botrytis fabae*: The Causal Agent of Chocolate Spot with Respect to Leaf Position

Mahmoud, H. El-Komy[1,2*], Amgad A. Saleh[1,3] and Younes Y. Molan[1]
[1]*Department of Plant Protection, College of Food and Agricultural Sciences, King Saud University, Kingdom of Saudi Arabia*
[2]*Plant Pathology Institute, Agriculture Research Center (ARC), Egypt*
[3]*Agricultural Genetic Engineering Research Institute (AGERI), Agriculture Research Center (ARC), Egypt*
[*]For correspondence: malkomy@ksu.edu.sa

Abstract

Plants have evolved different defense mechanisms to combat pathogen attacks. In this study, detached leaf assays were conducted to estimate the influence of leaf position (leaf age) on the development of chocolate spot disease, caused by *Botrytis fabae*, in the lower, middle and upper leaves of five faba bean (FB) cultivars with different levels of resistance/susceptibility. Two components of resistance in terms of lesion diameter and spores per lesion were used to evaluate the disease intensity. To evaluate plant defense response, oxidative burst and phenol-oxidizing enzymes were assayed. The results indicated that regardless of the resistance level of the FB cultivar, lower (older) leaves were more severely infected by the chocolate spot pathogen than upper (younger) ones. A comparison of the defense-response behavior of the FB leaves revealed that the chocolate spot pathogen induced lipid peroxidation and the production of reactive oxygen species, peroxidase, and polyphenol oxidase 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. Younger leaves exhibited significantly higher oxidizing enzyme activity and lower oxidative stress than older ones. These variations in the levels of defense compounds could explain the differences in leaf resistance. The extreme differences in disease development recorded in upper and lower leaves of all FB cultivars suggest that assessing resistance using leaves from the middle positions would be the most efficient and reliable for evaluating the resistance/susceptibility of FB plants to *B. fabae*.

Keywords: *Botrytis fabae*; Disease development; Leaf age; Plant defense; *Vicia faba*

Introduction

Faba bean (FB) (*Vicia faba* L.) is an important temperate legume crop being used as a source of protein in human diets and fodder for animals, in addition to its ecological role of increasing the available nitrogen content in the biosphere (Köpke and Nemecek, 2010). Fungal diseases of FB are very destructive causing considerable yield losses. Chocolate spot disease, caused by *Botrytis fabae* Sard., is an economically important fungal disease that damages FB leaves, limiting photosynthetic activity and hence reducing FB production globally (Torres *et al*., 2004). The deployment of resistant FB varieties is an efficient strategy for controlling chocolate spot disease and promoting the development of sustainable agriculture (Rhaïem *et al*., 2002; Bouhassan *et al*., 2004a). Detached leaflet assays have been widely used for initial screening of large numbers of FB varieties, thereby eliminating highly susceptible accessions before conducting costly field tests (Khalil and Harrison, 1981; Hanounik and Maliha, 1986; Hanounik and Robertson, 1988; Porta-Puglia *et al*., 1994). These assays also seemed to be useful for identifying the components of partial resistance or evaluating the effect of different factors on the response of FB plants against *B. fabae* (Bouhassan *et al*., 2003,2004b; Villegas-Fernández *et al*., 2012).

Resistance to pathogenic infections can vary over the life of a plant. In many plant-pathogen interactions, the degree of resistance depends on the genotype, growth stage, and leaf age of the host plant (Dolar, 1997; Turechek and Stevenson, 1998; Chang and Hwang 2003; Visker *et al*., 2003; Rodriguez *et al*., 2006; Basandrai *et al*., 2007; Develey-Rivïere and Galiana, 2007; Coelho and Valério, 2009). The difference in resistance to pathogens according to tissue age is age-related resistance (ARR) (Kus *et al*., 2002; Zeier, 2005; Ando *et al*., 2009; Al-Daoud and Cameron, 2011). Many previous studies reported that the resistance to chocolate spot disease is affected by the development of FB plant. Heilbronn and Harrison (1989) investigated the effect of FB leaf age on the pathogenicity of *B fabae*. They demonstrated that the oldest leaves developed more lesions than the youngest ones. Further, Bouhassan *et al*. (2004b) found that in addition to changes in resistance to

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B. fabae at different plant growth stages, older and younger leaves of the same FB plant varied in their level of resistance irrespective of the age of the plant. On the other hand, Villegas-Fernández *et al*. (2012) showed that the greater susceptibility to chocolate spot of older FB leaves in comparison to younger ones is not a general phenomenon but seems to be genotype-specific.

In response to pathogenic fungal invasions, plant tissues are generally able to mount a range of defense responses that restrict the growth, replication and spread of the pathogens and eventually can destroy them (Tarred *et al*., 1993; Weigend and Lyr, 1996; Nawar and Kuti, 2003; Shetty *et al*., 2008). However, little work has reported about the effect of FB leaf age on the production of plant defense-associated compounds. Heilbronn and Harrison (1989) reported that young FB leaflets infected with *B. cinera* accumulated more phytoalexins than old ones. Tarred *et al*., (1993) and Nawar and Kuti (2003) also showed that after inoculation with *B. fabae*, the production of the wyerone acid (a phytoalexin) and peroxidase increased in resistant FB cultivars than susceptible ones post inoculation with *B. fabae*, in both whole plants and detached leaves, irrespective of leaf age. The objective of this study was to investigate the defense-associated compounds involved in age related resistance and to optimize screening techniques for chocolate spot pathogen-resistant FB cultivars, by studying the effect of leaf position determining foliage age with regards to its effect on (1) disease development in five FB cultivars exhibiting different levels of resistance to the chocolate spot pathogen, and (2) the production of and variation in, an oxidative burst and phenol-oxidizing enzymes in FB leaves upon infection with the pathogen.

Materials and Methods

The present study was carried out in 2010–2011 at the Plant Protection Department, Faculty of Food and Agriculture Sciences, King Saud University, Kingdom of Saudi Arabia.

Plant Materials and Growth Conditions

Five FB cultivars exhibiting different levels of resistance to *B. fabae* were used in this study: Giza Blanka (resistant), Giza 461 (resistant), Sakha 2 (moderately resistant), Giza 2 (susceptible) and Giza 429 (susceptible). The FB cultivars were obtained from the Field Crop Research Institute, Agricultural Research Center, Giza, Egypt. The FB seeds were planted in 20 cm-diameter pots filled with a mixture of sterilized soil, peat moss and sand (1: 1: 1; v: v: v) and kept in an environmentally controlled greenhouse at 24±2°C with a 16 h photoperiod. The germinated seedlings were thinned to five plants per pot.

Preparation of the Fungal Inoculum

An aggressive single-spored *B. fabae* isolate recovered from a diseased FB plant exhibiting symptoms of chocolate spot disease was used in the pathogenicity tests carried out in this study. The fungal isolate was grown on potato dextrose agar plates and incubated at 25°C for 7–10 days. To induce sporulation, the fungal culture was transferred to FB leaf extract medium as described by Leach and Moore (1966) for 10 days at 20–22°C. Conidia were collected by washing plates with sterile water and the resulting spore suspension was adjusted to 3×10^6 conidia mL^{-1} using a hemocytometer (Bouhassan *et al*., 2003,2004b).

Disease Development Evaluation

At the beginning of the flowering stage (7-week-old plants), ten FB plants per cultivar were sampled. At this stage, the average number of leaves per plant of the FB cultivars used in this study was 9 $plant^{-1}$. The 1st, 2nd and 3rd leaves were considered as the lower leaves, the 4th, 5th and 6th as the middle ones, whereas the upper leaves were the 7th, 8th and 9th leaf. For each sampled FB plant, leaflets from lower (P1), middle (P2) and upper positions (P3) were selected. In the laboratory, the detached leaflets were grouped according to their position and placed in Petri dishes containing wet tissue papers (4 leaflets $plate^{-1}$) and inoculated with one centrally deposited droplet of 20 µL spore suspension (3×10^6 conidia mL^{-1}). Then, the dishes containing inoculated leaflets were incubated at 20°C. After 24 h of incubation, spore suspension droplets were blotted with tissue paper to discourage bacterial growth. Afterward, the dishes were transferred to a growth chamber with a 14 h photoperiod at 20°C. Disease development was recorded based on an average on 25 leaflets per leaf age and FB cultivar as described by Bouhassan *et al*. (2004a,b) by measuring two components of resistance: (1) lesion diameter in cm at 5 days post-inoculation (dpi) and (2) pathogen spore production expressed as the number of conidia per leaflet at 10 dpi. For consistency, the disease development evaluation experiments were conducted twice.

Plant Defense Response Assays

Oxidative burst: At the beginning of the flowering stage (7-week-old plants), 12 FB plants (2 plants pot^{-1}) from each cultivar were inoculated by spraying to run-off with a conidial suspension (3×10^6 conidia mL^{-1}) of *B. fabae* supplemented with 0.05% Tween 20. The inoculated plants were placed in a moist chamber in a greenhouse at 24±2°C with a 16 h photoperiod. The oxidative burst, including reactive oxygen species (ROS) activity and lipid peroxidation, was evaluated in selected leaflets according to their position on the stem at 0, 12, 24, 36, 48 and 72 h hpi (2 FB plants per cultivar for each time interval). The control FB plants were treated with sterilized water only. This experiment was conducted twice for reproducibility.

To assess the accumulation of ROS, the amount of superoxide anion (O_2) was determined according to the

method described by Doke (1983) with some modifications. FB leaflet tissue (4 replicates of 1 g each) were immersed in a mixture containing 0.05% (w/v) nitroblue tetrazolium, 10 mM potassium phosphate buffer (pH 7.8), and 10 mM NaN_3 (4 mL of mixture g^{-1} leaf tissue). One h after incubation, tubes containing 1 mL tissue extracts were heated to 85°C for 15 min and then cooled, after which the absorbance at 580 nm was measured. ROS activity was expressed as the change in absorbance at 580 nm g^{-1} fresh weight.

Lipid peroxidation was determined by measuring 2-thiobarbituric acid-reactive substances (TBARS) according to Polkowska-Kowalczyk *et al*. (2004) with some modifications. FB leaflet tissue (4 replicates of 1 g each) were homogenized in 5% (w/v) trichloroacetic acid (1 mL g^{-1} leaf tissue) and then centrifuged at 10,000 ×*g* for 10 min at 4°C. A total volume of 600 µL of 0.3% (w/v) sodium dodecyl sulfate, 0.25% (w/v) TBA in 50 mM NaOH and 6% (v/v) HCl were added to the 400 µL cell extract. After heating at 80°C for 40 min, TBARS were extracted with 1 mL of n-butanol. The specific activity of the organic phase was measured at a wavelength of 532 nm ($\boldsymbol{A}_{532}$), and the nonspecific activity was measured at a wavelength of 600 nm ($\boldsymbol{A}_{600}$). Lipid peroxidation was expressed as $\boldsymbol{A}_{532}$-$\boldsymbol{A}_{600}$ g^{-1} fresh weight.

Phenol-oxidizing Enzymes: The activities of the phenol-oxidizing enzymes peroxidase (PO) and polyphenol oxidase (PPO) were evaluated in excised leaflets according to their positions on the stem at 0, 24, 48 and 72 hpi (2 FB plants per cultivar for each time interval) as described previously for the evaluation of the oxidative burst. The control FB plants were treated with sterilized water only. This experiment was conducted twice for reproducibility.

PO activity was determined according to Ramanathan *et al*. (2001). FB leaflet tissue (4 replicates of 1 g each) were immersed in liquid nitrogen, ground into a powder using a mortar and pestle, which was then suspended in 100 mM phosphate buffer (pH 6.0) (1 mL g^{-1} leaf tissue). The solution was centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant containing the enzyme extract was collected. A 500 µL volume of 50 mM pyrogallol and 100 µL of enzyme extract were mixed in a 1 ml Jenway 6705 cuvette (Bibby Scientific Limited, Staffordshire, UK). The reference cuvette contained 100 µL of inactivated enzyme extract (by boiling) and 500 µL of 50 mM pyrogallol. To initiate the reaction, 100 µL of 1% hydrogen peroxide (V/V) were added to each sample cuvette, and the PO activity was measured within 30 s at 420 nm. PO activity was expressed as absorbance at $\boldsymbol{A}_{420}$ nm g^{-1} fresh weight.

PPO activity was determined by measuring the oxidation of catechol at 575 nm as described by Houssien and Sabra (2005) with some modifications. FB leaflet tissue (4 replicates of 1 g each) were immersed in liquid nitrogen, ground into a fine powder using a mortar and pestle and then suspended in 100 mM borate buffer (pH 9.0) (1 mL g^{-1} leaf tissue). The solution was centrifuged at 10,000 × *g* for 20 min at 4°C and then the supernatant containing the enzyme extract was collected. The reaction mixture consisted of 500 µL of borate buffer, 200 µL of *p*-aminobenzoic acid, 200 µL of 50 mM catechol and 100 µL of enzyme extract, which were mixed in a 1 mL Jenway 6705 cuvette (Bibby Scientific Limited, Staffordshire, UK). The reference cuvette contained 100 µL of inactivated enzyme extract (by boiling). PPO activity was measured at 575 nm after 1 h incubation in a shaker water bath at 40°C and the PPO activity was expressed as the change in absorbance at $\boldsymbol{A}_{575}$ nm g^{-1} fresh weight.

Data Analysis

All experiments were conducted using the factorial model of a completely randomized experimental design. In the disease development evaluation experiments, two factors (FB cultivars × leaf positions) with 25 replicates per treatment were examined. In the plant defense response assays, three factors (FB cultivars × leaf positions × time intervals) with four replicates per treatment were analyzed. Data of spore production were square root [sqrt (x + 0.5)] transformed prior to analysis (Gomez and Gomez, 1984). All experiments were conducted twice for reproducibility. Levene's test was used to cheek the data for homogeneity of variance, and indicated that the data of the two experiments could be combined (Gomez and Gomez, 1984). Statistical analyses were performed using SAS Version 9.1 software (SAS Institute Inc., 2003). Analyses of variances were performed for all datasets. All data were presented as mean values (average of two experiments) and the statistical significance was indicated at $P < 0.05$. The correlation coefficient (r) between the means of lesion diameter and spore production was calculated using MSTAT-C software.

Results

Disease Development

Detached leaf assays were conducted to estimate the effect of leaf position (leaf age) on the development of chocolate spot disease in five FB cultivars: Giza Blanka, Giza 461, Sakha 2, Giza 2 and Giza 429. Based on visual observations, the main characteristic of a resistant reaction was the fact that FB leaflets remained green and healthy up to 5 dpi. During this period, symptoms were restricted to a few dark brown hypersensitive spots (2–3 dpi). In case of the susceptible reaction, the FB leaflets were characterized by necrosis that developed into expanding and maceration lesions within 3–5 dpi. The fungus colonized the entire leaflet of susceptible plants and produced spores within 5–7 dpi on the leaflet surface (Fig. 1).

The factorial analysis of variance for lesion diameter and spore production revealed significant effects for FB cultivars, leaf positions and their interaction on the disease

Table 1: The effect of leaf position on disease development as expressed as lesion diameter (cm) in the leaflets for five FB cultivars displaying different levels of resistance/susceptibility to *B. fabae* inoculation at 5 dpi

Cultivars	Diameter of lesions (cm)*			M^a
	Leaf position			
	Lower	Middle	Upper	
Giza Blanka (R)	0.88	0.56	0.44	0.62 C
Giza 461 (R)	1.00	0.48	0.35	0.61 C
Sakha 2 (MR)	1.28	0.67	0.48	0.81 C
Giza 2 (S)	2.30	1.60	0.87	1.59 A
Giza 429 (S)	1.80	1.14	0.66	1.20 B
M^b	1.45 a	0.88 b	0.56 c	

Table 2: The effect of leaf position on disease development expressed as spore production leaflet^{-1} for five FB cultivars displaying different levels of resistance/susceptibility to *B. fabae* inoculation at 10 dpi

Cultivars	Number of conidia leaflet^{-1} (1×10^4)*			M^a
	Leaf position			
	Lower	Middle	Upper	
Giza Blanka (R)	3.7 (2.02)	3.9 (2.07)	1.6 (1.40)	3.1 D (1.83)
Giza 461 (R)	6.5 (2.62)	4.7 (2.10)	1.6 (1.38)	4.3 C (2.19)
Sakha 2 (MR)	7.0 (2.69)	4.9 (2.28)	2.1 (1.59)	4.7 C (2.10)
Giza 2 (S)	9.6 (3.16)	6.1 (2.56)	2.0 (1.48)	5.9 B (2.40)
Giza 429 (S)	9.6 (3.14)	6.9 (2.70)	3.9 (2.06)	6.8 A (2.64)
M^b	7.3 a (2.73)	5.3 b (2.38)	2.2 c (1.58)	

The letters in parentheses indicate the response of the FB cultivars to chocolate spot pathogen infection: (R) resistant, (MR) moderately resistant and (S) susceptible; The values in parentheses represent the transformed data of the number of conidia using square-root transformation; *Mean values are average of two experiments with 25 replicates for each treatment per experiment; **M^a**=Main effect of leaf position; **M^b**=Main effect of FB cultivars; $L.S.D_{0.05}$ for interaction (based on the transformed data)= 0.371; The values followed by the same lowercase or uppercase letters are not significantly different at $P < 0.05$

development ($P \leq 0.001$). Based on the components of resistance values, cultivars Giza Blanka and Giza 461 displayed high resistance to *B. fabae*. The FB cultivar Sakha 2 displayed moderate resistance. The lowest resistance was displayed by the susceptible FB cultivars Giza 2 and Giza 429 (Tables 1, 2). In general, there was a proportional relationship between the lesion size and number of spores (r= 0.873; *P*=0.001).

The main effect of leaf position showed that chocolate spot lesions were significantly larger on the lower leaves (1.45 cm) than on the middle (0.88 cm) and upper leaves (0.56 cm) (Table 1). After 10 days of inoculation, the numbers of *B. fabae* conidia were significantly higher on the lower leaves (7.3×10^4 conidia leaflet^{-1}) than on the middle (5.3×10^4 conidia leaflet^{-1}) and upper leaves (2.2×10^4 conidia leaflet^{-1}) (Table 2).

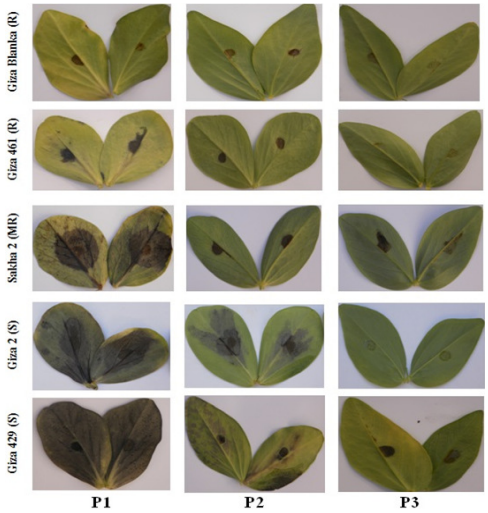

Fig. 1: The responses of the lower (P1), middle (P2), and upper (P3) leaflets of five FB cultivars to *B. fabae* at 5 dpi

The letters in parentheses indicate the response of the FB cultivars to chocolate spot pathogen infection: (R) resistant, (MR) moderately resistant and (S) susceptible

Regardless of the degree of resistance among the FB cultivars, the disease development on leaves in the same plant varied according to the position of the leaves on the stem (Tables 1, 2). In upper leaves, no significant differences in lesion diameter values were detected between the resistant cultivars (Giza Blanka and Giza 461) and the susceptible cultivar Giza 429. Additionally, spore production values of the susceptible cultivar Giza 2 did not differ significantly from the resistant ones (Tables 1, 2). In lower and middle leaves, the components of resistance values were able to differentiate between the resistant and susceptible cultivars. However, extreme values of lesion diameter and spore production (minima and maxima) in the upper and lower leaves of all cultivars were recorded (Tables 1, 2).

Plant Defense Response

Oxidative Burst: The changes in oxidative burst activities in FB leaves from different positions were determined after different periods (0, 12, 24, 36, 48, and 72 hpi) of inoculation with *B. fabae* (Fig. 2, 3 and 4). Results illustrate that regardless of the resistance levels of the FB cultivars, inoculation with *B. fabae* resulted in an increase in oxidative burst activities in the leaf tissues compared with the activities in un-infected leaves (Fig. 2). No significant effects of the interactions between FB cultivars x leaf position or FB cultivars x leaf position x time-course analyses were detected for the oxidative burst activities.

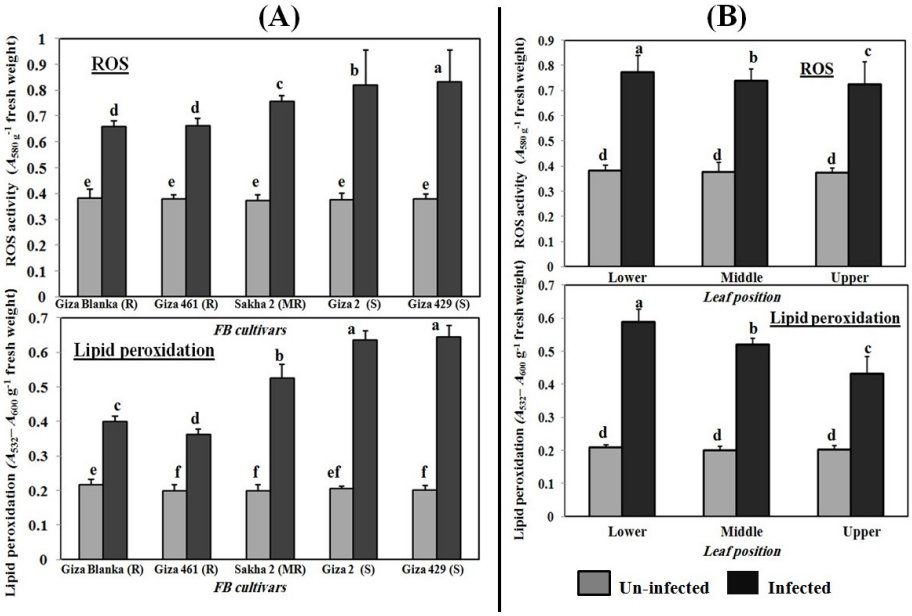

Fig. 2: Overall effect of FB cultivars (A) and leaf position (B) on the changes in the ROS activity and lipid peroxidation in response to infection with *B. fabae*. FB plants were inoculated with sterilized water (un-infected) or with *B. fabae* (infected) and the changes in the ROS activity and lipid peroxidation were assayed during 72 h post inoculation. The experiment was conducted twice and the data for the two experiments were combined

The letters in parentheses indicate the response of the FB cultivars to chocolate spot pathogen: resistant (R), moderately resistant (MR), and susceptible (S). In overall effect of FB cultivars, each value is the means of 144 replicates {3 leaf position x 6 time intervals x with 8 replicates per treatment (of two experiments)}. In overall effect of leaf position, each value is the means of 240 replicates {5 FB cultivars x 6 time intervals x 8 replicates per treatment (of two experiments)}. In each plant defense response within the same overall effect, bars with the same letter are not significantly different at $P< 0.05$. Error bars represent the standard deviations of the mean

The activities of oxidative burst varied significantly among FB cultivars ($P \leq 0.001$). However, the susceptible cultivars Giza 429 and Giza 2 showed the highest increase in oxidative burst activities, whereas the resistant cultivars Giza Blanka and Giza 461 displayed the lowest activities (Fig. 2). Leaf position was also associated with highly significant differences in oxidative burst activities ($P \leq 0.001$). The ROS activity and level of lipid peroxidation in the lower (older) FB leaves were significantly higher (0.772 and 0.589, respectively) than those in the upper (younger) ones (0.724 and 0.432, respectively) (Fig. 2).

Time course of the changes in oxidative burst activities in FB leaf tissues after inoculation with *B. fabae* are presented in Fig. 3, 4. Results showed that ROS accumulated rapidly in the infected leaf tissues of the FB cultivars and reached the maximum levels within 24–36 hpi then declined significantly. Whereas in the susceptible cultivars, ROS activities gradually increased with time and reached the maximum levels within 36–72 hpi (Fig. 3). In addition, the increment rate of lipid peroxidation was higher in susceptible than in the resistant FB cultivars (Fig. 4).

Regardless of the resistance levels, the oxidative burst generation profiles were different between the younger and older leaves in the all FB cultivars (Fig. 3). The upper leaves displayed significant increases in ROS activity and lipid peroxidation after 12 hpi that peaked (0.818–1.23 and 0.471–0.745, respectively) within 24–36 hpi. Conversely, the largest significant increases in ROS activity (1.22–1.52) and lipid peroxidation (0.618–0.990) were detected in the lower leaves at 36–72 hpi (Fig. 3). These results indicated that ROS peaked earlier in resistant leaf tissues than in susceptible ones following the inoculation with *B. fabae*. No significant changes in the oxidative burst activities were detected with time in uninfected leaf tissues (data not shown).

Phenol-oxidizing Enzymes: The changes in activities of the PO and PPO in lower, middle and upper leaves of the FB cultivars were determined after different periods (0, 12, 24, 48 and 72 hpi) of inoculation with *B. fabae*. Results showed that the effects of the interactions between FB cultivars x leaf position or FB cultivars x leaf position x time-course analyses were insignificant for the activities of PO and PPO.

The activities of these enzymes were differed significantly between various FB cultivars at $P \leq 0.001$. The highest increases in PO and PPO activities were detected in the resistant cultivar Giza Blanka (1.37 and 0.815, respectively), followed by Giza 461 (1.23 and 0.928, respectively) (Fig. 5). Susceptible cultivars Giza 2 and Giza 429 had the lowest increases in PO and PPO activities (Fig. 5).

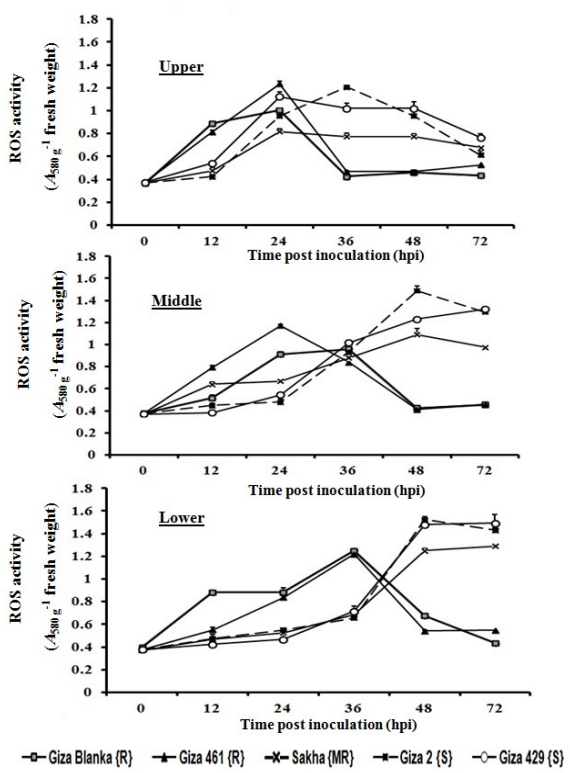

Fig. 3: Time course of the changes in ROS activities in the lower, middle, and upper leaves of five FB cultivars in response to infection with *B. fabae*

The letters in parentheses indicate the response of the FB cultivars to chocolate spot pathogen: resistant (R), moderately resistant (MR), and susceptible (S). Each value is the means of eight replicates (of two experiments). L.S.D$_{0.05}$ = 0.067. Error bars represent the standard deviations of the mean. No significant changes in the activities were detected with time in uninfected leaf tissues (data not shown)

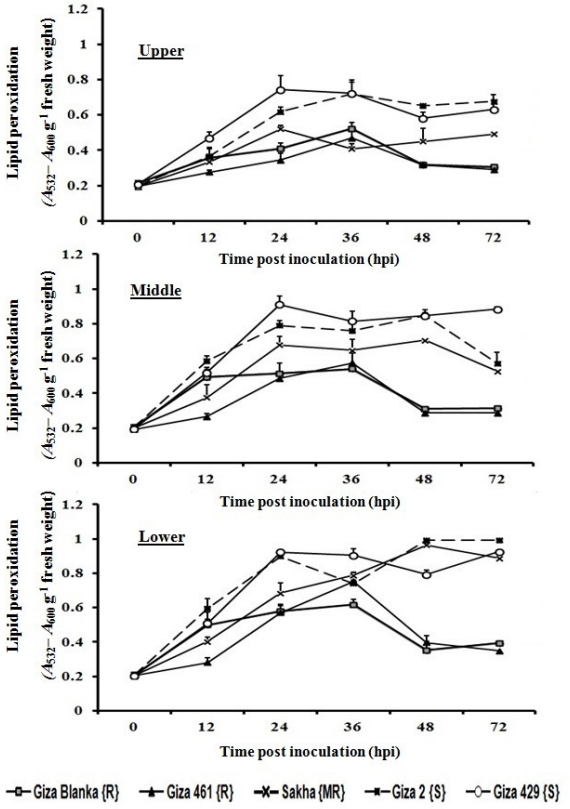

Fig. 4: Time course of the changes in lipid peroxidation in the lower, middle, and upper leaves of five FB cultivars in response to infection with *B. fabae*

The letters in parentheses indicate the response of the FB cultivars to chocolate spot pathogen: resistant (R), moderately resistant (MR), and susceptible (S). Each value is the means of eight replicates (of two experiments). L.S.D$_{0.05}$=0.068. Error bars represent the standard deviations of the mean. No significant changes in the activities were detected with time in uninfected leaf tissues (data not shown)

Regardless of the resistance levels of the FB cultivars, significant differences in phenol-oxidizing enzymes activities at $P \leq 0.001$ were observed in FB leaves according to their positions (Fig. 5). As response to inoculation with *B. fabae*, high increases in PO and PPO activities were detected in the upper (younger) leaves (1.38 and 0.851, respectively). Moderate levels of PO and PPO activities were detected in the middle leaves (1.087 and 0.776, respectively). However, in the lower leaves, the PO and PPO activities were 0.836 and 0.607, respectively (Fig. 5).

The time-course of phenol-oxidizing enzymes activities in FB leaf tissues after inoculation with *B. fabae* are shown in Fig. 6, 7. Results showed that PO and PPO activities increased significantly with time in all FB cultivars and were higher at all time-course analyses in resistant and moderately resistant than susceptible cultivars. Among the FB cultivars, the production rate of PO and PPO was more rapid and higher in the upper and middle leaves than in the lower ones (Fig 6, 7). No significant changes in the phenol-oxidizing enzymes activities were detected with time in uninfected leaf tissues (data not shown).

Discussion

Plant resistance is believed to be correlated with plant age. Generally, the resistance/susceptibility increases as plants get older. It has been observed that resistance to chocolate spot disease is affected by FB plant development (Heilbronn and Harrison, 1989; Bouhassan *et al*., 2004b). However, the effects of tissue age on the expression of plant defense-associated compounds have not been studied in detail. The objective of this work was to quantify the effect of leaf position (leaf age) on resistance/susceptibility responses in five FB cultivars with different levels of resistance to *B. fabae*. In detached leaf assays, the resistance response measured in terms of lesion diameter and spore production was correlated with the resistance level of FB cultivars. The highest lesion diameter and spore production values were observed in susceptible cultivars Giza 2 and Giza 429, whereas the lowest values were observed in resistant cultivars Giza Blanka and Giza 461. These results support the relationship between the expression of these

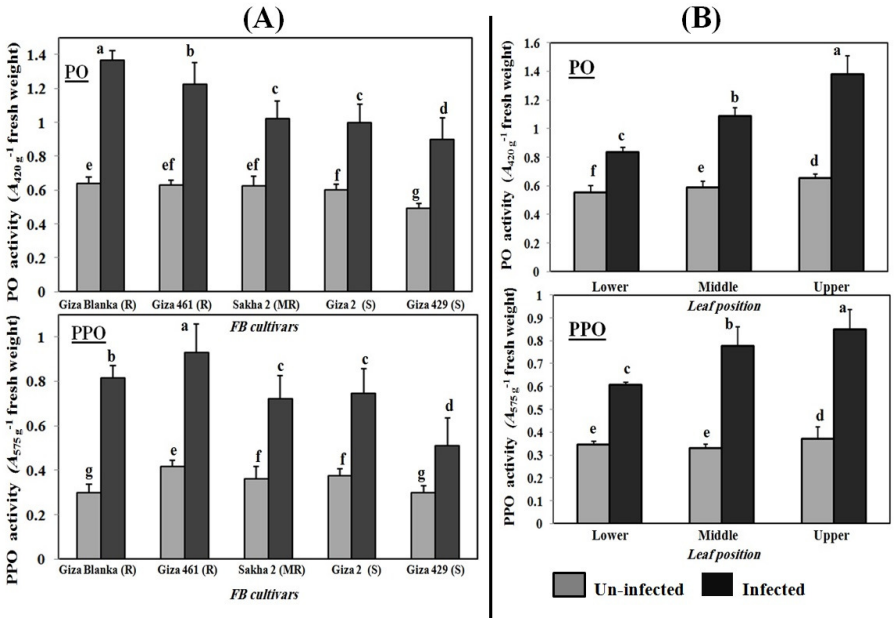

Fig. 5: Overall effect of FB cultivars (A) and leaf position (B) the changes in the activities of PO and PPO in response to infection with *B. fabae*. FB plants were inoculated with sterilized water (un-infected) or with *B. fabae* (infected) and the changes in the activities of PO and PPO were assayed during 72 h post inoculation. The experiment was conducted twice and the data for the two experiments were combined

The letters in parentheses indicate the response of the FB cultivars to chocolate spot pathogen: resistant (R), moderately resistant (MR), and susceptible (S). In overall effect of FB cultivars, each value is the means of 96 replicates {3 leaf position x 4 time intervals x 8 replicates per treatment (of two experiments)}. In overall effect of leaf position, each value is the means of 160 replicates {5 FB cultivars x 4 time intervals with x 8 replicates per treatment (of two experiments)}. In each plant defense response within the same overall effect, bars with the same letter are not significantly different at $P < 0.05$. Error bars represent the standard deviations of the mean

resistance components and the resistance/susceptibility to chocolate spot disease (Bouhassan *et al*., 2003, 2004a and 2004b).

Regardless of the resistance levels of the FB cultivars, the disease development on leaves in the same plant varied according to their position on the stem. Chocolate spot lesions were significantly larger on the lower (older) leaves than on the upper (younger) ones. After 10 days of inoculation, the numbers of *B. fabae* conidia were significantly higher on the lower leaves than on the middle and upper ones. Based on these results, the *B. fabae* isolate was more aggressive in causing chocolate spot disease on older leaves than on younger ones. These results indicate that leaf position (leaf age) played a significant role in disease development. These results were consistent with many previous studies, which lead to the conclusion that leaf age is a major factor affecting the response of FB against chocolate spot pathogen (Creighton *et al*., 1986; Heilbronn and Harrison, 1989; Bouhassan *et al*., 2004b). However, Villegas-Fernández *et al*. (2012) reported that FB resistance/susceptibility against *B. fabae* seems to be genotype-specific rather leaf age dependent. The variability in resistance/susceptibility to a pathogen according to leaf age has been reported in other host-pathogen systems such as: potato/*Phytophthora infestans* (Visker *et al*., 2003), potato/*Alternaria solani* (Rodriguez *et al*., 2006) and chickpea/*Ascochyta rabiei* (Basandrai *et al*., 2007).

Detached leaflet assays have been used to assess reaction of FB varieties against *B. fabae* (Khalil and Harrison, 1981; Hanounik and Maliha, 1986; Hanounik and Robertson, 1988; Porta-Puglia *et al*., 1994; Bouhassan *et al*., 2003, 2004a and 2004b). Generally, there was a positive correlation between the detached leaf assays and field evaluations for both susceptible and resistant cultivar, but for moderate resistant cultivars no clear correlation (Bouhassan *et al*., 2003) has been reported. In the present study, the interaction between FB cultivars and leaf positions significantly affect on the disease development. Regardless of the resistance levels, extreme values of lesion diameter and spore production (minima and maxima) in the upper and lower leaves of all FB cultivars were recorded. In the upper leaves, components of resistance values of the resistant cultivars did not differ significantly from that of susceptible ones. When assessing leaves from middle or lower position, the lesion diameter and spore production values were able to differentiate between the resistant and susceptible cultivars. However, lower leaves may not provide a fair discrimination between resistant and susceptible cultivars because these leaves may show compatible reactions in resistant plants. Consequently, the use of FB leaves from the middle positions would be the most efficient for evaluating the resistance/susceptibility

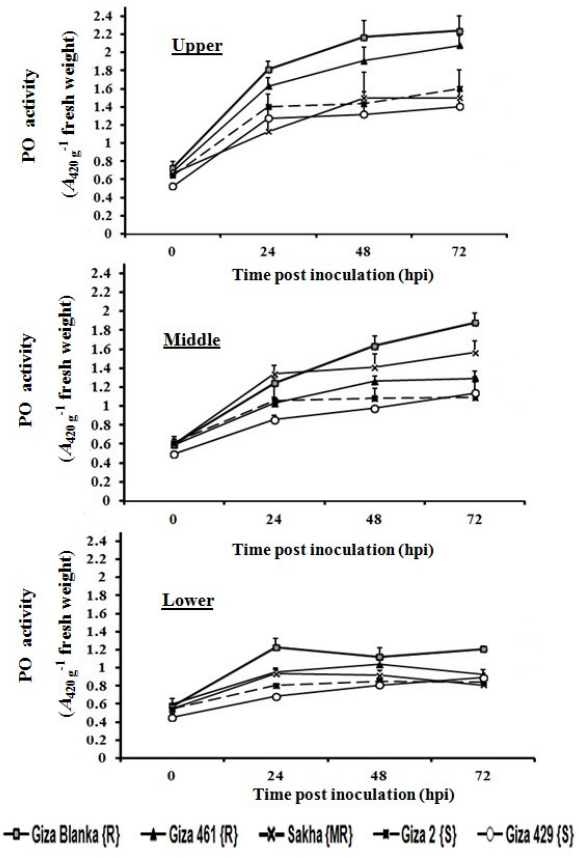

Fig. 6: Time course of the changes in the activities of PO in the lower, middle, and upper leaves of five FB cultivars in response to infection with *B. fabae*

The letters in parentheses indicate the response of the FB cultivars to chocolate spot pathogen: resistant (R), moderately resistant (MR), and susceptible (S). Each value is the means of eight replicates (of two experiments). L.S.D$_{0.05}$= 0.145. Error bars represent the standard deviations of the mean. No significant changes in the activities were detected with time in uninfected leaf tissues (data not shown)

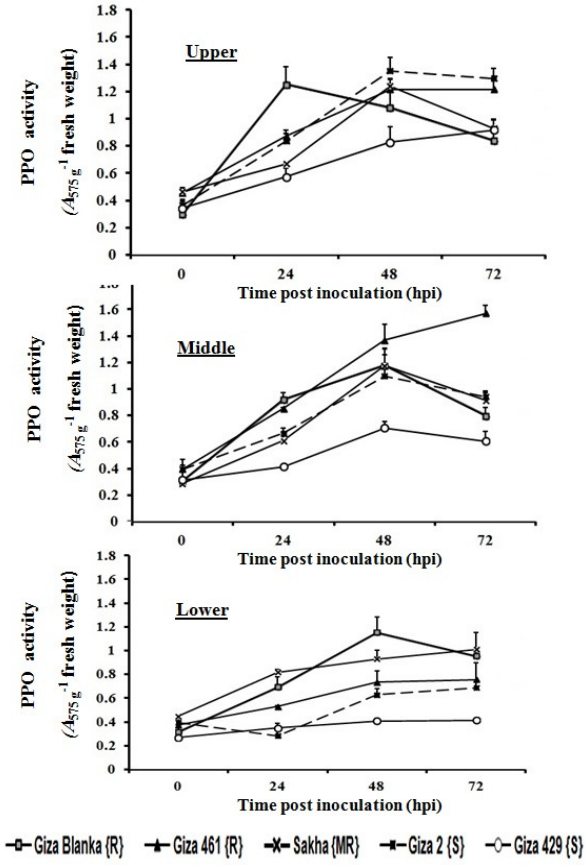

Fig. 7: Time course of the changes in the activities of PPO in the lower, middle, and upper leaves of five FB cultivars in response to infection with *B. fabae*

The letters in parentheses indicate the response of the FB cultivars to chocolate spot pathogen: resistant (R), moderately resistant (MR), and susceptible (S). Each value is the means of eight replicates (of two experiments). L.S.D$_{0.05}$ =0.101. Error bars represent the standard deviations of the mean. No significant changes in the activities were detected with time in uninfected leaf tissues (data not shown)

against *B. fabae*. Moreover, random leaf sampling for resistance assessments against *B. fabae* should be avoided to minimize the effect leaf position. These findings are similar to those reported by Rodriguez *et al.* (2006) in case of early blight disease on potato caused by *A. solani*.

In response to the pathogenic fungi invasions, plant tissues are generally able to mount a spectrum of defense responses. Studying the changes in the expression and variation of these defense responses may clarify the effect of leaf position (leaf age) on the resistance/susceptibility of FB leaves to *B. fabae*. One of the earliest and most effective plant defense reactions against pathogens is the production of ROS compounds such as O^{-2}, hydrogen peroxide (H_2O_2), and hydroxyl radicals during the so-called oxidative burst (Malolepsza and Urbanek, 2000; Hancock *et al*., 2001; Malolepsza, 2005; Shetty *et al*., 2008). The importance of ROS generation has been attributed to their role in many defense processes, including direct antimicrobial actions, reinforcement of the plant cell wall (e.g., cell wall lignification and cross-linking of cell wall structural proteins), phytoalexin production, the hypersensitive response (HR), and the development of systemic acquired resistance (Malolepsza and Urbanek, 2000; Malolepsza, 2005; Shetty *et al*., 2008). The ROS role in the defense mechanism against necrotrophic fungi (e.g. *Botrytis* spp.) remains controversial (Asselbergh *et al*., 2007). There are some studies demonstrated a positive effect of ROS on disease-resistance mechanisms (Malolepsza and Urbanek, 2000; Malolepsza, 2005; Unger *et al*., 2005). Conversely, other studies revealed that higher generation of ROS assists necrotrophic pathogens to colonize host tissues (von Tiedemann, 1997; Govrin and Levine, 2000; Mayer *et al*., 2001; Schouten *et al*., 2002; Able, 2003; El-Komy, 2014). The formation of ROS in plant tissues results in hypersensitive cell death, which enables the invasion and spread of necrotrophs in the dead host tissues (von

Tiedemann, 1997; Govrin and Levine, 2000; Mayer *et al*., 2001; Schouten *et al*., 2002; Able, 2003). These studies are consistent with our results, which showed that after inoculation with *B. fabae*, the susceptible cultivars Giza 429 and Giza 2 showed the highest oxidative burst activities, whereas the resistant cultivars Giza Blanka and Giza 461 displayed the lowest activities. In all FB cultivars, the accumulation of ROS was higher in older leaves, which are highly susceptible to infection, than in the younger ones. These results indicated that the oxidative burst activities significantly varied among FB cultivars and leaf positions. The higher levels of ROS correlated with susceptibility of FB leaf tissues to infection with *B. fabae*.

Interestingly, the oxidative burst generation profiles were different between the younger and older leaves. In the younger leaves, ROS activities reached their highest levels in the early stages of infection (24–36 hpi). In contrast, the highest increases in ROS activities in the older leaves were detected during late stages of infection (36–72 hpi). A similar trend was noticed in resistant FB cultivars where higher levels of ROS activities reached in early stages of infection compared with susceptible ones. These results indicated that ROS peaked earlier in resistant leaf tissues than in susceptible ones following the inoculation with *B. fabae*. In addition, we observed that the timely hyperinduction of ROS was associated with the hypersensitive reaction that appeared in younger leaves or the necrosis that transformed into expanded and macerated lesions in older leaves. These observations suggest that high accumulation of ROS in younger leaf tissues during the earlier stages of infection may have a direct fungitoxic effect on the pathogen (e.g. delaying spore germination), which allows the plant to initiate a cascade of defense reactions. In contrast, the high accumulation of ROS in older leaves enhances the fungal colonization of plant tissues. Malolepsza and Urbanek (2000) showed that *B. cinerea* conidia were sensitive to H_2O_2, and higher concentrations of it inhibited fungal mycelial growth. However, the time of H_2O_2 production is critical. Malolepsza and Urbanek (2000) observed that if the germination of *B. cinerea* conidia did not stop at the early infection stages, then high concentrations of H_2O_2 could not inhibit the mycelial growth inside the host tissues. Asselbergh *et al*. (2007) concluded that the timing of ROS production and accumulation was crucial in their defensive role against *B. cinerea* development in tomato plants. The same was observed for the interaction of *B. fabea* with FB plants expressing different levels of resistance against this pathogen (El-Komy, 2014). These results indicate that the variation in the resistance/susceptibility of older and younger leaves against the chocolate spot pathogen was most likely due to differences in the timing and quantity of ROS production in the FB leaf tissues.

The infection of FB leaves by *B. fabae* resulted in oxidative damage, as indicated by increases in the propagation of lipid peroxidation. There was a positive relationship between lipid peroxidation in the FB leaves and the development of chocolate spot disease. Such results are in agreement with those reported by Weigend and Lyr (1996) who reported that the inoculation of FB leaflets with *B. cinerea* resulted in enhanced lipid peroxidation in the diseased tissues during the early stages of infection. As a response to infection, the inoculated leaves from the lower and middle positions displayed significantly higher levels of lipid peroxidation than the upper leaves. These results indicate that low oxidative damage detected in the younger leaves explained the attenuation of disease development in these leaf tissues. The works of Grovin and Levine (2000) and van Baarlen *et al*. (2004) support these previous results, as they demonstrated that the induction of programmed cell death facilitates *Botrytis* spp. invasion and may in fact be essential for successful infection of their host plants.

The role of these oxidizing enzymes in plant-pathogen interactions has long been associated with the formation of toxic metabolites and structural barriers against invading pathogens (Letcher, 1970; Weigend and Lyr, 1996). A number of studies indicated that PPO is involved in the oxidation of polyphenols into quinines (antimicrobial compounds) and the lignification of plant cell walls during microbial invasion. Tarred *et al*. (1993) reported that increases in PO activity enhanced the cell wall lignification in response to chocolate spot pathogen infection and consequently restricted its penetration. In this study after infection of FB leaves with *B. fabae*, we observed that PO and PPO activities increased significantly with time in all FB cultivars and were higher at all time-course analyses in resistant and moderately resistant than susceptible cultivars. The highest increases in PO and PPO activities were detected in resistant cultivars Giza Blanka and Giza 461. These results suggest that the expression of phenol-oxidizing enzymes can be associated with the resistance to *B. fabae*. Nawar and Kuti (2003) also reported that PO activity could be used as a biomarker to evaluate the resistance reactions in FB plants against chocolate spot disease.

Additionally, there were highly significant differences in oxidizing enzyme activities in FB leaves according to their positions. As a response to inoculation with *B. fabae*, the production rate of PO and PPO was more rapid and higher in the younger (upper) leaves than older (lower) ones. These results indicated that the gradual increases in resistance from the lower to upper FB leaves are most likely due to the differences in the activities of phenol-oxidizing enzymes.

Conclusion

The results of this study support the contention that FB resistance to chocolate spot disease is age-related. Regardless of the resistance levels of the FB cultivars, the disease development on the leaves of the same plant varied

significantly according to their position (age) on the stem. Lower (older) leaves were more susceptible than upper (younger) ones. During the FB *B. fabae* interaction, the production of plant defense-associated compounds in leaves is not static, but instead, governed (timing and accumulation) by the physiological maturity of the plant. These variations in the production of defense compounds can explain the variations in leaf resistance. Based on these results, we recommend that breeders and pathologists consider leaf position during the evaluation of newly developed resistant cultivars against chocolate spot pathogen to avoid selection errors that may lead to contradictory results.

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