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Protein Analysis and Peroxidase Isozymes as Molecular Markers for Resistance and Susceptibility of Sunflower to *Macrophomina phaseolina*

S.S. ABOSHOSHA, S.I. ATTA ALLA†, A.E. EL-KORANY† AND E. EL-ARGAWY†
Plant Pathology Department, Faculty of Agriculture, El-Shatby and †Damanhour, Alexandria University, Egypt
Corresponding author's e-mail: drelkorany125@yahoo.com

ABSTRACT

In this study, the sunflower cv. Giza 102 was found to be the most tolerant (no plant death) to *Macrophomina phaseolina* isolates recovered from El-Behera governorate, while cv. H20 was extremely susceptible (90.6% plant death), while cvs. Giza53, Auroflor and Vidoic showed intermediate susceptibility. Such resistance and susceptibility were reflected in different protein patterns, peroxidase activity and isozymes. Four protein bands were recognized in the analyzed healthy seedlings of the resistant cv.Giza102, while the susceptible cv. H20, showed only two protein bands. Cultivars with intermediate susceptibility showed 3-4 bands and number of bands increased with increasing degree of tolerance. The protein bands with intermediate molecular weight of 20-100 kDa only appeared in the most resistant cvs, *i.e.*, cv. Giza102 and cv. Giza 53, while none of the susceptible (cv. H20) or intermediately susceptible cultivars (Auroflor & Vidoic) exhibited protein bands in this range. The inoculation with *M. phaseolina* resulted in a greater number of protein bands (7-9 bands), but bands of 67-138 kDa were frequent (4 bands) in the resistant cv. Giza102 compared to only 1-2 bands for the susceptible and the intermediately susceptible ones. On the other hand, a consistently higher peroxidase activity over three tested intervals (r = 0.940 - 0.943) was recorded in the resistant cv. Giza102 compared to the susceptible cv. H20 with more pronounced activity in the analyzed seedlings compared to the analyzed mature plants. The inoculation with *M. phaseolina* resulted in an even higher peroxidase activity with more pronounced activity in the resistant cultivar particularly 48 h and 72 h after inoculation in the analyzed seedlings and mature plants, respectively. It was also notable that peroxidase activity in the analyzed mature plants was always lower compared to the analyzed seedlings, which could explain susceptibility of sunflower in the late stage to *M. phaseolina* infection. Meantime, three peroxidase isozymes designated P17, P77 and P88 were recognized in the healthy seedlings of the resistant cv. Giza102, while only two (P98 & P120) revealed in the analyzed mature plants. The same isozymes were detected in the susceptible cv. H20. However, the resistant cv. Giza102 showed a consistently higher isozyme activity over three intervals of investigation. P88 isozyme showed the most consistent reaction, which could be a suitable tool for recognizing resistance and susceptibility of sunflower to *M. phaseolina*. These results revealed the validity of protein analysis, peroxidase activity and peroxidase isozymes pattern as genetic markers for resistance and susceptibility in sunflower to *M. phaseolina*.

Key Words: Disease resistance; Isozyme; *M. phaseolina*; Proteins; Peroxidase activity; Sunflower

INTRODUCTION

Involvement of protein components and peroxidase activity in plant diseases resistance has been documented in several plant patho-systems (Tornero *et al*., 2002; Martin *et al*., 2003; Carvalho *et al*., 2006). Different kinds of proteins were found to play certain roles in the plant defense mechanism and the resistance to plant pathogens (Belkhadir *et al*., 2004). Peroxidase was recorded (Sulman *et al*., 2001) as one of the first enzymes responding and providing fast defense against plant pathogens. Infection with plant pathogens led to an induction in Peroxidase activity in plant tissues and a greater increase was recorded in resistant plants compared to the susceptible ones (Mydlarz & Harvell, 2006).

Infiltration of leaves with a commercial preparation of peroxidase or even its direct activation was recorded to protect susceptible plants against plant pathogens (Converso & Fernandez, 1996). Such antifungal effect of peroxidase was suggested to be due to certain peroxidase isozymes (Caruso *et al*., 2001). Induction of peroxidases due to pathogen interaction has been studied in a number of patho-systems, where novel peroxidases appeared and higher activity was recorded in resistant cultivars compared to susceptible ones (Flott *et al*., 1989; Kirstensen *et al*., 1999; Ramanathan *et al*., 2001). The present study therefore, was conducted to investigate the association of the protein and the peroxidases in sunflower to resistance and susceptibility to *M. phaseolina* and validity of such components as genetic markers to recognize resistance and susceptibility to the infection with *M. phaseolina*.

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MATERIALS AND METHODS

The tested of *Macrophomina phaseolina* isolates. Sixteen *M. phaseolina* isolates represented different regions in El-Behera governorate were used in the present study. The isolates were recovered from different fields in El-Behera governorate, where sunflower was grown during the 2003 and 2004 growing seasons and identified according to Dhingra and Sinclair (1978) and Barnett and Hunter (1987).

Varietal reaction of sunflower cultivars to *M. phaseolina* isolates. Five sunflower cultivars widely grown in Egypt were tested in a pot experiment for their relative resistance and susceptibility to the above mentioned *M. phaseolina* isolates. The tested cultivars were Giza53, Giza102, Auroflore, Vidoic and H20. Seeds of the tested cultivars were obtained from the Seed Dept., Ministry of Agric., El-Giza, Egypt. Seeds were surface disinfested with 2% sodium hypochlorite, rinsed in sterile distilled water and sown in 25 cm plastic pots filled with autoclaved clay and sand (1:1, v:v). Five replicate pots each was sown with two seeds of the tested sunflower cvs were prepared for each tested isolate. Plants were watered as needed and treated according to the normal agricultural practices. The 60-day-old plants were, then, stem inoculated, 1 cm above the lowest node, with 7-day-old PDA culture of the tested *M. phaseolina* isolates using inoculation technique based on Day and MacDonald (1995). Inoculated plants were transferred to growth cabinet with 18 h fluorescent light and 28 ± 2ºC. Control plants were inoculated with PDA free of the fungus and treated in the same way. Three weeks after inoculation, developed lesions were measured (in cm) as the longitudinal bark necrosis below and above the site of inoculation (Day & MacDonald, 1995). Percentage of plant death and percentage of reduction in plant head diameter relative to the control were also determined three weeks after inoculation (Gamal El-Din *et al*., 1984). Re-isolation was conducted to insure the association of the tested isolates with the developed disease.

Molecular Characterization of Resistant and Susceptible Sunflower Cultivars to *M. phaseolina* Isolates

Total protein analysis: plants, pathogens and inoculation. Seeds of the most resistant and the most susceptible sunflower cultivars, detected in the varietal reaction test, were brought from the Seed Dept., Ministry of Agric., El-Giza, Egypt and sown in plastic pots and treated as above mentioned. Two weeks later, developed seedlings were stem inoculated 1 cm above the soil surface (Day & MacDonald, 1995) using 7-day-old PDA culture of the most vigorous *M. phaseolina* isolate detected in the varietal reaction test. Inoculated plants were transferred to growth cabinet with 18 h fluorescent light, 28 ± 2ºC, and treated as the normal agricultural practices. Ten seedlings were prepared for each sunflower cultivar.

Protein extraction and electrophoresis. One week after inoculation, seedlings (healthy/infected) were taken off the pots and site of inoculation with the 1 cm surrounding area was discarded to eliminate interference of the fungal protein. Rest of the seedling was homogenized in 62.5 mm Tris-HCl buffer (pH 6.8), filtrated through four layers of muslin cloth, centrifuged at 10,000 x g for 10 min and extracted for soluble protein according to Kope *et al*. (1998). Protein extracts were then electrophoresed with standard protein marker on polyacrylamide gel (10%) according to Laemmli (1970) methods of the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained according to Hames and Rickwood (1990) and scanned with Video Copy Processor P65E (Appligene) for their banding patterns. Quantitative determination of the resolved protein polypeptide content of bands was carried out using the molecular dynamic image Quant V3.3 program (Appligene) according to El-Agamy (2000). All chemicals were Bio-Rad products, USA.

Peroxidase analysis: plants, pathogens and inoculation. Most resistant and the most susceptible sunflower cvs. detected in the varietal reaction test were grown up to two stages of growth; 15 days old seedling and 60-day-old plants. Plants were prepared and inoculated with *M. phaseolina* as described above using the most vigorous *M. phaseolina* isolate detected in the varietal reaction test (Day & MacDonald, 1995).

Exraction of peroxidases. Samples for peroxidase analysis were taken 0, 24, 48 and 72 after inoculation from the area surrounding site of inoculation in stem of the mature 60-day-old plants as well as the seedling shoot above site of inoculation. Total peroxidase was extracted according to Srivastava (1987) and Anderson *et al.* (1995). Samples were homogenized in 0.01 M sodium phosphate buffer (pH 6.0) as 2 mL buffer g^{-1} fresh weight and filtered through four layers of muslin cloth. The filtrates were then centrifuged at 10,000 x g at 4ºC for 20 min and the supernatant served as the enzyme source.

Total peroxidase activity. Total peroxidase activity was determined using spectrophotometer (Analytic Jana 40) according to Srivastava (1987) by using 0.05 M pyrogallol. Reference sample contained inactivated boiled enzyme extract. The 1% hydrogen peroxide (v/v) was used to initiate the reaction and change in absorbance was recorded at 30s intervals. Total peroxidase activity was determined in four replicates and expressed as the mean absorbance at 470 nm/g f.w./min.

Peroxidase isozymes fractionation and activity. Peroxidase isozymes were analyzed using the native polyacrylamide gel electrophoresis (native-PAGE) 10%, according to Pan *et al*. (1991). The gels were run for 2 h at 10ºC and 30 mA in a Bio-Rad vertical electrophoresis unit. Gels were stained according Ramanathan *et al.* (2001) and scanned with the video copy processor P65E (Appligene) for the peroxidase isozyme bands. Peroxidase isozymes were designated and coined by their migration position (mm of the origin line) on the gel according to Manjunatha *et al*. (2003). Bands intensity *i.e*., their specific activity, was determined with Helena 24-P densitometer at 470 nm and

expressed as units of isozyme activity/mg isozyme protein/min., where one unit equal 0.001 absorbance at 470 nm/min under 26 ± 2ºC room temperature (Omidiji *et al*., 2002). All chemicals were Bio-Rad products.

Statitical analysis. The obtained data were statistically analyzed using the American SAS/STAT Software, version 6 (SAS Institute Inc., Cary, USA) and means were compared by the least significant difference test (LSD). Correlation and regression analyses were conducted using the data analysis program on the Microsoft Office Exel, 2003.

RESULTS

Sunflower varietal reaction to *M. phaseolina*. The sunflower cv. Giza 102 exhibited the highest tolerance to the infection with sixteen isolates represented *M. phaseolina* population of El-Behera governorate (Table I). Percentage of plant death for cv. Giza102 was as low as 0% with 7.3% reduction in plant head diameter and 2.6 cm mean stem lesions incited in the varietal reaction test. The cv.H20, however, exhibited the highest susceptibility as 90.6% plant death, 60.4% reduction in plant head diameter, and 10.9 cm mean lesions were recorded. The cv.Giza53 was moderately susceptible as 0% plant death was recorded with 21.9% reduction in plant head diameter and 4.1 cm mean stem lesions. The cv. Auroflor and cv. Vidoic of sunflower were more susceptible as 59.3%, 70.3% plant death, 41.5%, 48.7% reduction of head diameter and 6.1 cm, 5.3 cm means stem lesions were recorded for the two cultivars, respectively (Table I).

Molecular Characterization of Resistant and Susceptible Sunflower Cultivars to *M. phaseolina* Isolates

Protein analysis. A protein banding pattern with 2-4 polypeptide bands was noted in the healthy (non-inoculated) sunflower cvs (Fig. 1; Table II). However, the most tolerant (resistant) cv. Giza102 exhibited four protein bands, while the most susceptible cv. H20 exhibited only two bands. Sunflower cvs with intermediate susceptibility to *M. phaseolina i.e.*, cv. Giza53, cv. Auroflor, cv. Vidoic, showed 3-4 bands and number of bands mostly increased with increasing the level of tolerance. Bands with molecular weight of 20-100 kDa appeared only in the most tolerant cv. Giza102 and cv. Giza 53, while none of the susceptible (cv. H20) or the intermediately susceptible (cv. Auroflor; cv. Vidoic) cvs exhibited such bands. On the other hand, inoculation of the same sunflower cvs with *M. phaseolina* resulted in a protein banding pattern consisted of a greater number of 7-9 polypeptide bands. However, the most resistant sunflower cv. Giza102 exhibited 4 polypeptide bands with 67-138 kDa range versus only 1-2 bands of this kDa range for the susceptible and the intermediately susceptible sunflower cvs (Table II). The polypeptide content of the resolved bands, however, did not exhibit any distinct trend in this respect (Table II).

Peroxidase Analysis

Total peroxidase activity. High correlation coefficient (r = 0.94 - 0.99) was revealed with the regression analysis conducted, which explains the strong relationship between time and total peroxidase activity (Fig. 2). Total peroxidase activity in the healthy (non-inoculated) most resistant sunflower cultivar (cv. G102) tested was consistently higher over the three intervals of the investigation (r = 940-943), in

Table I. Varietal reaction of sunflower cultivars inoculated with isolates of *Macrophomina phaseolina* recovered from diseased sunflower plants collected from different fields in El-Behera governorate during the 2003 and 2004 growing season

Sunflower cvs	Varietal reaction		
	Stem Lesion (cm)[2]	% Plant Death[1]	% Head Diameter Reduction[1]
H20	10.9	90.6	60.4
Auroflor	6.1	59.3	41.5
Vidoic	5.3	70.3	48.7
Giza 53	4.1	0.0	21.9
Giza102	2.6	0.0	7.3
LSD at 0.05%	1.3	19.6	11.7

Values are means of sixteen *M. phaseolina* isolates represented the 2003; 2004 growing seasons and the different regions of sunflower cultivation in El-Behera Governorate, Egypt, five replicate pots for each isolate were prepared. [1]percentage relative to the non-inoculated control. [2]values over the control.

Fig. 1. SDS-PAGE (10%) of protein in seedlings of five sunflower cultivars non-inoculated (a), and inoculated (b), with *M. phaseolina* (isolate 32/2004). Gels were scanned with the video copy processor P65E (Appligene) for their banding patterns. MW= molecular weight in kilo Dalton (kDa), Std.= standard protein marker, anode is towards the bottom of the photo.

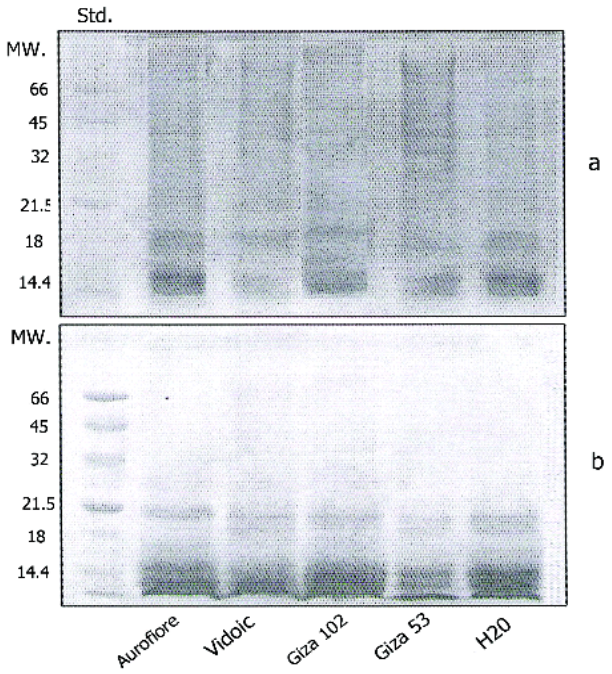


Table II. Protein banding pattern and percentage of the polypeptide content of the developed bands in sunflower cultivars non-inoculated and inoculated with *Macrophomina phaseolina* (isolate 32/2004)

MW (kDa)	Sunflower cultivars									
	Non-inoculated (healthy control)					Inoculated				
	H20	Auro	Vidoic	Giza 53	Giza 102	H20	Auro	Vidoic	Giza 53	Giza 102
138							0.9			
129										2.7
128										
127										
126								13.8		
125						10.6				
123							16.2		17.7	8.1
120		71.8								
115						2.6				
100										
96										15.5
80				71.5						
76										2.7
31									31.8	
27								39.0		
26						31.7	44.8			
25									10.3	
24								13.9		35.5
23						8.6			3.2	
20					93.2	5.4			6.2	
19	81.1		87.0							
18				13.8						
17							6.5			
16		19.4				6.4				
15						4.5		5.3	6.8	10.9
14						12.6	11.6	9.1	8.6	6.2
13			4.4				3.3	6.5		6.5
12	18.8			7.6			3.9		6.5	
11					2.4	17.3	12.4	11.2		11.2
10		8.68	6.8	4.5	1.7				8.0	
9					2.5					
8			1.65							
3										
N B	2	3	4	4	4	9	8	7	9	9

N B= number of bands. MW= molecular weight of the developed protein bands in kilo Dalton (kDa). Values are percentage of the polypeptide content of the developed bands quantified in sunflower seedlings using the molecular dynamic image Quant V3.3 program (Appligene). Auro= cv. Auroflor.

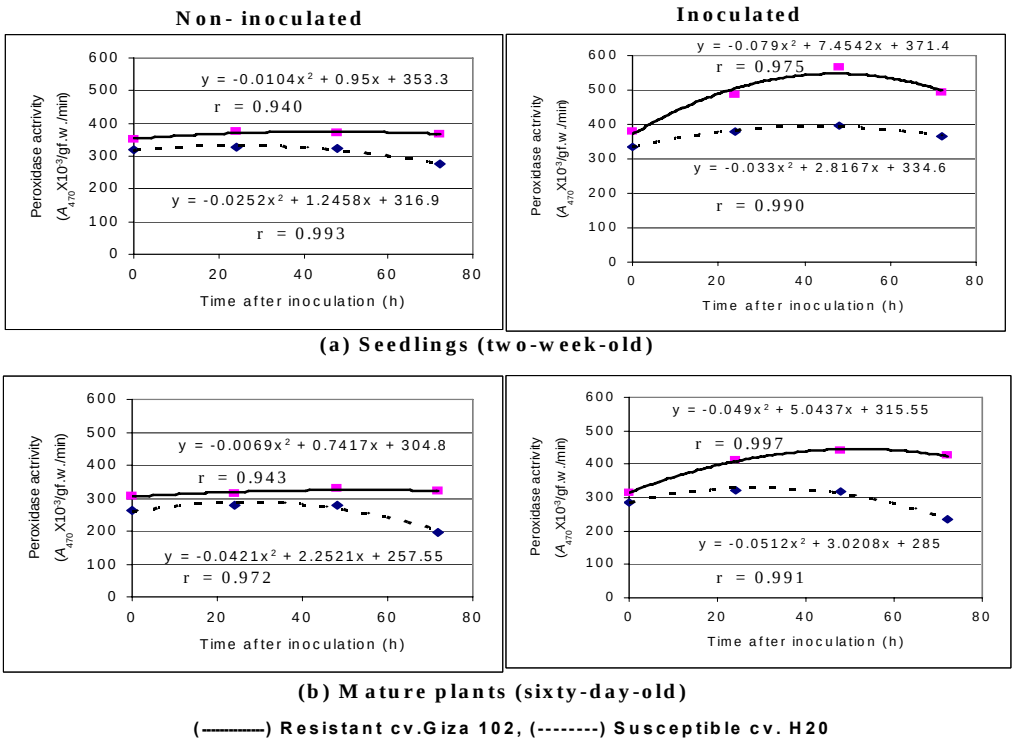
both seedlings and mature plants analyzed, compared to the susceptible cv.H20 (Fig. 2). This was most pronounced at 72 h of the investigation. On the other hand, an apparent increase in peroxidase activity was recorded in both resistant and susceptible sunflower cvs after inoculation with *M. phaseolina* (isolate 32/2004) over the three intervals of investigation. The rate of increase, however, was more pronounced in the analyzed resistant cv. Giza 102 compared to susceptible cv.H20 particularly 48 and 72 h after inoculation in the analyzed seedlings and mature plants, respectively (Fig. 2). It was also notable that peroxidase activity in the inoculated mature plants was always lower than peroxidase activity in the inoculated seedlings over three intervals of investigation (Fig. 2).

Peroxidase isozymes. Three peroxidase isozymes designated P17, P77, and P88 were recognized in the analyzed healthy (non-inoculated) seedlings of the resistant cv. Giza 102 of sunflower (Fig. 3). Meantime, only two peroxidase isozymes designated P98, and P120 were recognized in the analyzed healthy mature plants. The same isozyme pattern was recognized in susceptible healthy cv. H20. However, the resistant cv. Giza 102 exhibited a consistently higher isozyme activity over the three intervals of investigation (r = 0.774 - 0.99) compared to the susceptible cv. H20 (Fig. 4). The same isozyme pattern was revealed after inoculation with *M. phaseolina* (isolate 32/2004). However, activity of the peroxidase isozymes tended to be higher particularly for P88 isozyme. The P88 isozyme showed the most consistent reaction over the three intervals of the investigation (r= 0.969 - 0.988) and showed a considerable higher activity in the resistant cv. Giza 102 of sunflower compared to the susceptible cv. H20 (Fig. 4).

DISCUSSION

Screening conducted for resistance to *M. phaseolina* among five of the most widely grown sunflower cultivars in Egypt revealed that cv. Giza 102 was the most tolerant (0% plant death) to *M. phaseolina* endemic isolates in El-Behera governorate while cv. H20 was extremely susceptible (90.6% plant death). The cv. Giza53, cv. Auroflor, and cv. Vidoic, however, showed intermediate susceptibility. Such resistance and susceptibility were reflected in different protein patterns, peroxidase activity and isozymes in the different sunflower cvs. The most tolerant (resistant) cv.Giza102, exhibited four protein bands in the analyzed healthy seedlings, while the most susceptible cv.H20,

Fig. 2. Total peroxidase activity in resistant (Giza102) and susceptible (H20) cultivars of sunflower non-inoculated and inoculated with *M. phaseolina* (isolate 32/2004) over three intervals of investigation.



exhibited only two bands. Sunflower cvs with intermediate susceptibility showed 3-4 bands and number of bands mostly increased with increasing the degree of tolerance. Besides, the protein bands with molecular weight of 20-100 kDa only appeared in the most resistant cvs, *i.e.* cv.Giza102 and cv. Giza 53, while none of the susceptible or the intermediately susceptible cvs exhibited protein bands of this kDa range. On the other hand, inoculation of sunflower cvs seedlings with *M. phaseolina* resulted in a greater number of protein bands (7-9 bands), however, bands with the molecular weight of 67-138 kDa were frequent (4 bands) in the most resistant sunflower cv. Giza102, versus only 1-2 bands for the susceptible and the intermediately susceptible sunflower cultivars. A greater number of polypeptide protein bands detected in the resistant cultivars indicated special disease resistance proteins, pathogenesis-related proteins, signalings, or even novel isozymes (Van Loon, 1985; Morimoto *et al*., 1999; Caruso *et al*., 2001; Iqbal *et al*., 2002; Tornero *et al*., 2002; Martin *et al*., 2003; Belkhadir *et al*., 2004; Carvalho *et al*., 2006; Ibrahim, 2006).

Comparison of total peroxidase activity in the healthy (non-inoculated) resistant cv.Giza102 and the susceptible cv.H20 of sunflower showed a consistently higher peroxidase activity in the resistant cultivar over the three intervals of the investigation (r = 940-943) compared to the susceptible one with more pronounced activity in the analyzed seedlings compared to the analyzed mature plants.

Fig. 3. Native-PAGE (10%T) of peroxidase isozymes in (a) seedlings and (b) mature plants of resistant (Giza102) and susceptible (H20) cultivars of sunflower non-inoculated and inoculated with *M. phaseolina* (isolate 32/2004), 48h after inoculation. Isozymes (P17, P77, P88, P98, P120) were coined with their migration position. Anode is towards the bottom of the photo

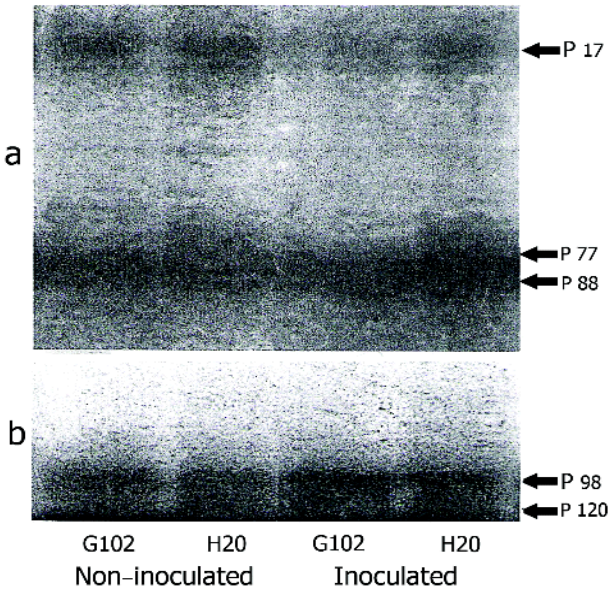
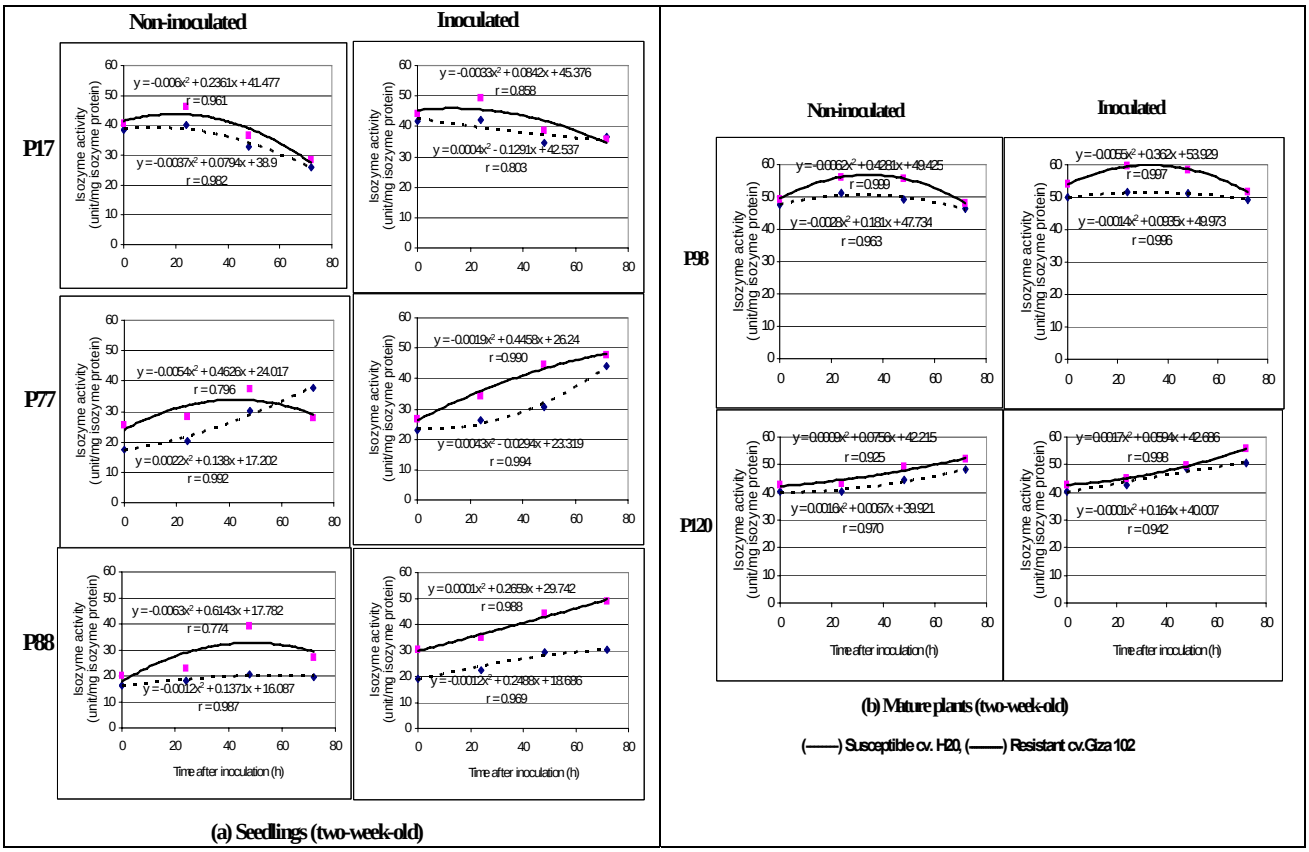


Fig. 4. Peroxidase isozymes activity in (a) seedlings and (b) mature plants of resistant (Giza102) and susceptible (H20) cultivars of sunflower non-inoculated and inoculated with *M. phaseolina* (isolate 32/2004). The control (non-inoculated) was only inoculated with PDA disc free of the *M. phaseolina* tested fungus



The inoculation with *M. phaseolina* resulted in an even higher peroxidase activity (r = 0.97-0.99) with more pronounced activity in the resistant cultivar particularly 48 h and 72 h after inoculation in the analyzed seedlings and mature plants, respectively. It was also notable that peroxidase activity in the analyzed mature plants was always lower than peroxidase activity in the analyzed seedlings. These findings were consistent with the association of peroxidase with plant resistance as peroxidase is one of the first enzymes responding to plant pathogen with increasing its activity as a resistance specific reaction against plant pathogens. Earlier studies suggest that peroxidase activity could lead to oxidizing different phenolic acids to oxidized antifungal compounds or the direct inhibition of fungi (Wallace & Fry, 1995; Caruso *et al*., 2001). Besides, the reduction reported in peroxidase activity with plant aging (Regnier & Macheix, 1996), consistent with results of the present study, could explain the frequently observed sunflower susceptibility to *M. phaseolina* in the mature plant stage compared to the early plant stages (Pratt *et al*., 1998; Sulman *et al*., 2001; Carvalho *et al*., 2006; Mydlarz & Harvell, 2006).

Three peroxidase isozymes designated P17, P77 and P88 were recognized in the analyzed healthy seedlings of the resistant cv. Giza 102 of sunflower. Also, two more peroxidase isozymes designated P98, and P120, were detected in the analyzed resistant mature sunflower plants. The same five isozymes were recognized in the susceptible cv.H20. However, the isozymes differed in their activity, where the resistant cv. Giza 102 showed a consistently higher activity over the three intervals of investigation (r = 0.774 - 0.99) compared to the susceptible cv. H20. A similar isozyme pattern was identified after inoculation with *M. phaseolina* but with slightly higher isozymes activity partiqularly for P88 isozyme. Isozyme analysis, meantime, revealed that most of total peroxidase activity in the analyzed seedlings of the resistant cultivar tested was due to activity of the isozymes P77 and P88, while P17 had no contribution in this respect. On the other hand, it does not seem that neither P98 nor P120 had a major contribution in total peroxidase activity in the analyzed mature plants in view of shape of their regression curve. The P88 isozyme showed the most consistent reaction over the three intervals of the investigation (r= 0.96 - 0.98), which makes it, in particular, a suitable tool for the recognition of resistance and susceptibility in sunflower. The role of peroxidase isozymes in plant resistance was through their involvement in catalyzing a variety of reactions such IAA catabolism,

lignin biosynthesis, suberization of cell wall and as a H_2O_2 detoxification system in plant cell (Ye *et al*., 1990; Regnier & Macheix, 1996; Bernards *et al*., 1999; Morimoto *et al*., 1999; Golubenko *et al*., 2006). These data were consistent with Ramanathan *et al*. (2001), Taylor and Francis (2005) and Saikia *et al*. (2006), and supported the validity of protein analysis and the peroxidase isozymes as genetic markers for resistance and susceptibility of fungal disease(Gentzbittel, 1998; Tornero *et al*., 2002; Upadhyay *et al*., 2002; Wang & Ma, 2002; Martin *et al*., 2003, Golubenko *et al*., 2006).

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