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RESEARCH ARTICLE / *ARTIGO*

Association of oxidative stress components with resistance to flax powdery mildew

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ABSTRACT

Field trials were conducted in 2008/2009 and 2009/2010 growing seasons at Giza Agricultural Research Station to evaluate powdery mildew (PM) severity on nine flax cultivars. Tested cultivars could be classified into five distinct groups, i.e., highly susceptible (Corland and C.I. 2008), susceptible (Giza 7 and Marshall), moderately susceptible (Cass), moderately resistant (Dakota, Koto and Wilden) and resistant (Ottowa 770B). They showed considerable variation in PM severity ranging from 8.1 on Ottowa 770B to 97.0% infected leaves/plant on Cortland. Total soluble proteins, total phenols, peroxidase, polyphenoloxidase, ascorbic acid, tocopherol and malondialdehyde were determined in infected leaves of the tested cultivars. Pearson's correlation coefficient was calculated to measure the degree of association between PM severity and each biochemical component. All components showed significant ($P<0.05$) or highly significant ($P<0.01$) negative correlation with PM severity except MDA, which showed positive correlation ($P<0.01$). The results of the present study suggest that phenols and MDA in infected leaves could be used to assist the screening of resistant plants at early stages of powdery mildew development.

Key words: *Linum usitatissimum*, *Oidium lini*, ascorbic acid, lipid peroxidation, peroxidase, polyphenoloxidase, tocopherol.

INTRODUCTION

Powdery mildew (*Oidium lini* Ŝkoric) of flax (*Linum usitatissimum* L.) is currently considered the most common, conspicuous, widespread, and easily recognized foliar disease of flax in Egypt. Flax is grown for both seeds and fibers in the Nile Delta, particularly in the northern governorates. This area is characterized by the prevalence of warm, wet weather during the late period of flax growing season. Such weather favors epiphytotic spread of the disease when virulent isolates of the causal fungus occur. Over the last two decades, the importance of this disease has increased probably due to the appearance and rapid distribution of new races capable of attacking the previously resistant cultivars (Aly et al., 1994). Currently, resistance is not available in the commercially grown flax cultivars in Egypt (Aly et al., 2002). Therefore, in years when environmental conditions favor the development of the disease, foliar application of fungicides has become the only commercially available management practice for its control (Aly et al., 1994).

Although the fungus causing powdery mildew on flax in other countries has been reported as *Erysiphe polygoni* DC. Ex Merat (Nyvall, 1981). In Egypt, it has not been observed in its perfect stage. Therefore, in the present work the fungus will be referred to its imperfect (conidial) stage, i.e. *Oidium lini* Škoric (Muskett & Colhoun, 1947).

Extensive genetic variation in terms of PM resistance has been identified in some flax populations. For example, Prasad et al. (1988) evaluated 2822 linseed varieties for rust and PM resistance and could classify them according to the percentage of infected leaf area per plant. Only 24 of those lines were free from both rust and PM, and 17 showed multiple resistances (1-10% infected leaf area /plant). In addition, 38 genotypes were free from rust and resistant to PM. Basandrai et al. (1994) evaluated 200 indigenous and exotic flax genotypes for resistance to PM under field conditions. Twenty-four genotypes were free from infection, and 12 genotypes were resistant to PM and also possessed desirable plant height for fiber flax. Mahto et al. (1995) found a significant variability among 26 flax genotypes in resistance to PM. Eleven had above average stability and seven of these had high yields. Of 43 flax introductions evaluated in a two-year outdoor pot experiment, Mansour et al. (2003) found that only three introductions showed satisfactory levels of resistance in both years. Some of the other introductions were resistant in only one year, which indicated that their performance lacked stability.

It has been suggested that a variety of compounds contained in plant cells are involved in resistance or susceptibility to infection by pathogens. Among these are proteins (Strange, 2003), phenols (Agrios, 2005), ascorbic

acid (Vidyasekaran, 2008), tocopherol (Castle & Day, 1984), peroxidase (Agrios, 2005), polyphenoloxidase (Agrios, 2005) and malondialdehyde as indicator of lipid peroxidation (Göbel et al., 2003).

No information about the biochemical basis of resistance or susceptibility of flax to PM is available. Therefore, the objective of the present study was to investigate if the previously mentioned biochemical components are associated with resistance of flax to PM.

MATERIALS AND METHODS

Reactions of flax cultivars to PM

Field trials were conducted in 2008/2009 and 2009/2010 growing seasons at Giza Agricultural Research Station to evaluate PM severity on nine flax cultivars. The experiments consisted of a randomized complete block design of three replications (blocks). Plots of 6 m² (2 x3 m) consisted of ten rows spaced 20 cm apart. Seeds of each cultivar were sown by hand at a rate of 70 g/plot. Planting date was the first week of December. Flax cultivars were left for natural infection and disease severity was rated visually in the last week of April based on percentage of infected leaves/ plant in a random sample of 10 plants/ plot (Nutter et al., 1991). Disease severity was not rated based on percentage of infected leaf area because at this stage of disease development, surface of infected leaves was almost completely covered with fungal growth.

Chemical analysis

Random samples of infected leaves were used for chemical analysis at early stage of disease development when plants were 80 days old. At this age, the disease was in its detection threshold (Nutter et al., 1991). Samples were stored in deep freezer at -30ºC for two days. Biochemical components were determined as follows:

Total phenols

Soluble phenols in fresh samples were extracted according to Dihazi et al. (2003). 0.5 g of fresh samples weight were extracted with 80% cold methanol (v/v) for three times at 90ºC. The combined extract were collected and filtered through Whatman No.1 filter paper. After filtration, the filtrate was made up to 20 mL with cold methanol. A 0.5 mL volume of the extract was added to 0.5 mL folin-Cicalteu reagent and shacked well. The mixture was allowed to stand for 3 min and then one mL of saturated sodium carbonate solution (25 g Na_2Co_3 were dissolved in 1000 mL distilled water at 70–80ºC and cooled down and filtered) was added to the mixture and shaken vigorously. The mixture was allowed to stand for 60 min. and then, the optical density was measured with a spectrophotometer at 725 nm UV-Vis. The quantity of total phenolic compounds was calculated according to the standard curve of gallic acid (99.5%) and expressed as mg/100 g fresh weight.

Assay of peroxidase activity

Two grams of fresh sample were homogenized either in cold phosphate buffer (0.05 M at pH 6.5). The homogenate was centrifuged at 1000 rpm for 10 minutes. The pigments were removed from the supernatant by adsorbing on activated charcoal and filtered. The filtrate was completed to a known volume and used to determine enzymes and total soluble protein.

Peroxidase (EC 1.11.1.7) was assayed following the method of Kar & Mishra (1976) with slight modification. Five mL of the assay mixture contained 300 µM of phosphate buffer (pH 6.8), 50 µM catechol, 50 µM H_2O_2 and 1 mL of crude enzyme extract was prepared. After incubation at 25ºC for 5 minute, the reaction was stopped with the addition of 1 mL of 10% H_2SO_4. The colour was read at 430 nm and the enzyme activity was expressed as enzyme activity/g fresh weight /hour.

Assay of polyphenoloxidase activity

Polyphenoloxidase (EC 1.14.18.1) was assayed following the method described by Kar & Mishra (1976) with little modification; five mL of assay mixture containing 125 µM of phosphate buffer (pH 6.8) and 100 µM pyrogallol were added to 1 mL of crude enzyme extract. After incubation at 25ºC for 5min, the reaction was stopped with the addition of 1 mL of 10% H_2So_4 the colour intensity was read at 430 nm, and the enzyme activity was expressed as enzyme activity/g fresh weight/hour.

Total soluble protein

Alkaline tartarate reagent 20 g sodium carbonate and 0.5 g tartarate were dissolved in liter of 0.1 N NaOH. Ten µL of the protein sample were added to 5 mL of the working alkaline copper reagent, and was allowed to stand for 15 min. at room temperature. The dilution folin reagent (0.5 mL) was then mixed immediately with the mixture; and allowed to stand 30 min. at room temperature. The color intensity of samples was measured at 750 nm. The total soluble protein content in the supernatant was determined according to Lowry et al. (1951) the quantity of total soluble protein was calculated according to the standard curve of Bovine Serum Albumin and expressed as mg/g fresh weight.

Ascorbic acid

Two grams of fresh samples were ground in 6% trichloroacetic acid (TCA) and the extract filtered through filter paper and centrifuged at 1000 rpm for 20 min. The filtrate was made up to 10 mL with TCA. Ascorbic acid was determined as described by Mukherjee & Choudhuri (1983). Four mL of the extract was mixed with 2 mL of 2% dinitrophenyl hydrazine (in acidic medium) followed by the addition of drop of 10% thiourea (in 70% ethanol). The mixture was boiled for 15 min in a water bath and after cooling to room temperature, 5 mL of 80% (v/v) H_2SO_4 was added to the mixture at 0ºC (in an ice bath) the absorbance was recorded at 530 nm using spectrophotometer. The

concentration of ascorbic acid was calculated from a standard curve plotted with known concentration of ascorbic acid and expressed as µg/g fresh weight.

Tocopherol

The content of α-tocopherol was determined as described by Philip et al. (1954). Five hundred mg of fresh tissue was homogenized with 10 mL of a mixture of petroleum ether and ethanol (2:1.6 v/v) and the extract was centrifuged at 10.0 00 rpm for 20 min. Then, the supernatant was collected and used for estimation of α-tocopherol. One mL of extract, 0.2 mL of 2% 2.2-dipyridyl in ethanol was added and mixed thoroughly and kept in the dark for 5 min. the resulting red colour was diluted with 4 mL of distilled water and mixed well. The resulting colour in the aqueous layer was measured at 520 nm the α-tocopherol content was calculated using a standard graph made with a known amount of α-tocopherol. The results were expressed in µg/g fresh weight.

Lipid peroxidation

The content of malondialdehyde (MDA) was measured according to the method of Heath & Packer (1968). 0.5 g of fresh samples were homogenized in 10 mL of 6% trichloroacetic acid and then centrifuged at 10.000 rpm for 15 min. The supernatant (1 mL) was mixed with 4 mL of 0.5% thiobarbitric acid and then heated at 95ºC for 30 min. The non-specific absorbance at 600 nm was subtracted from the 532 nm absorbance. The absorbance coefficient of malondialdehyde was calculated by using the extinction coefficient of 155 mM/cm and expressed as a total MDA nmol/g fresh weight.

Statistical analysis

The experimental design of the field trials and the laboratory tests was randomized complete block with three replicates (blocks). Analysis of variance (ANOVA) of the data was performed with MSTAT-C. Duncan's multiple range test was used to compare cultivar means. Linear correlation coefficient (r) was calculated to evaluate the degree of association between levels or activities of biochemical components and PM severity ratings on the tested cultivars. Correlation analysis was performed with a computerized program (SPSS Version 13).

RESULTS

Environmental conditions in both 2008/2009 and 2009/2010 growing seasons were favorable for powdery mildew development resulting in 97.0% PM severity on the cv. Cortland, which is known as highly susceptible (A.A. Aly, *personal observations*). In general, the tested cultivars could be classified into five distinct groups, i.e. highly susceptible (Cortland and C.I. 2008), susceptible (Giza 7, and Marshall), moderately susceptible (Cass), moderately resistant (Dakota, Koto, and Wilden), and resistant (Ottowa 770 B). The cultivars showed considerable variation in PM severity ranging from 8.05 on Ottowa 770B to 97.02% on Cortland (Table 1).

Levels and activities of seven biochemical components were determined in leaves of nine flax cultivars varying in PM severity (Table 2). The levels and activities of the tested components varied among the cultivars. However, protein and peroxidase activity were the only components that did not show any significant difference among cultivars.

Levels and activities of all the tested components (Table 3) showed highly significant and positive correlations with each other (r value ranged from 0.776 to 0.970). MDA was a notable exception because it showed highly significant and negative correlations with all the components (r value ranged from -0.798 to -0.941, $P < 0.01$).

In the present study, MDA was the only component, which showed positive correlation ($r = 0.851$, $P < 0.01$) with PM severity (Table 3). The other components showed significant ($P < 0.05$) or highly significant ($P < 0.01$) negative correlation with PM severity. Total protein, phenols and MDA showed the highest correlation with PM severity.

DISCUSSION

In the present study, associations among the tested biochemical components in infected flax leaves and PM severity were identified (Table 3) and the relative strength of these association was measured by calculating

TABLE 1- Powdery mildew severity ratings on nine flax cultivars and their disease categories under field conditions in Giza in 2008/2009 and 2009/2010 growing seasons

Cultivar	Disease severity [a] (%)		Disease category [b]
Cortland	97.0	A	HS
C.I. 2008	95.9	A	HS
Giza 7	70.4	C	S
Marshall	64.7	C	S
Cass	48.5	D	MS
Koto	24.0	E	MR
Dakota	21.7	E	MR
Wilden	18.3	E	MR
Ottowa 770B	8.1	F	R

[a]Disease severity is the percentage of infected leaves/plant in a random sample of 10 plants/plot. Each value is the mean of two growing seasons and each season included three replicates. Percentage data were transformed into arcsine angles before carrying out the analysis of variance to normalize data and stabilize variance throughout the data range. Means followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

[b]Disease categories are highly susceptible (HS), susceptible (S), moderately susceptible (MS), moderately resistant (MR), and resistant (R).

TABLE 2 - Levels and activities of biochemical components in infected leaves of nine flax cultivars

Cultivar	Component															
	Total protein (mg g^{-1} fresh weight)		Phenols (mg g^{-1} fresh weight)		Ascorbic acid (µg g^{-1} fresh weight)		Tocopherol (µg g^{-1} fresh weight)		Malondialdehyde (MDA) (n mol g^{-1} fresh weight)		Peroxidase (activity h^{-1} g^{-1} fresh weight)		Polyphenoloxidase (activity h^{-1} g^{-1} fresh weight)	
Ottowa 770B	23.38	A	319.50	A	15.74	B-E	46.95	A-C	49.55	B	27.73	A-C	26.03	A
Dakota	22.63	A	306.70	A	27.65	A	37.89	D-F	42.67	B	27.32	A-C	18.60	B
Cass	21.45	A	244.30	BC	19.81	B	35.50	E-G	69.16	A	22.14	A-C	15.85	BC
Wilden	21.11	A	265.90	B	19.66	B	37.17	D-F	49.46	B	21.39	A-C	13.18	CD
Koto	20.81	A	219.10	CD	13.96	C-F	28.79	G-I	72.95	A	17.24	BC	11.51	DE
Marshall	19.76	A	217.20	CD	17.48	B-D	34.28	E-G	70.37	A	18.02	BC	11.22	DE
Giza 7	18.43	A	214.00	CD	18.23	BC	33.73	F-H	70.45	A	18.72	BC	12.56	DE
Cortland	16.66	A	195.60	D	12.64	D-F	26.06	I	81.72	A	14.58	C	5.45	G
C.I. 2008	18.72	A	191.20	D	12.41	D-G	26.47	HI	84.73	A	16.83	BC	6.90	FG

Each value is the mean of three replicates. Within a column, means followed by the same letter(s) are not significantly different ($P < 0.05$) according to Duncan's multiple range test. Determination of levels and activities of components was made when the plants were 120 days old.

TABLE 3 - Correlation between levels and activities of some biochemical components in infected leaves of nine flax cultivars and correlation between powdery mildew (PM) severity and each of these components

Levels and activities	Levels and activities of						
	Tocopherol	Phenols	MDA	Ascorbic acid	Total protein	Peroxidase	PM severity
Tocopherol							-0.756*
Phenols	0.903**[a]						-0.845**
MDA	-0.836**	-0.941**					-0.851**
Ascorbic acid	0.806**	0.892**	-0.911**				0.687*
Total protein	0.819**	0.894**	-0.816**	0.776*			-0.905**
Peroxidase	0.898**	0.970**	-0.890**	0.925**	0.899**		-0.768*
Polyphenoloxidase	0.951**	0.923**	-0.798**	0.807**	0.892**	0.938**	-0.801**

[a] Pearson's correlation coefficient (r) is significant at $P < 0.01$ (**) or $P < 0.05$ (*).

Pearson's correlation coefficient (r). However, one should keep in mind that the significant correlation does not necessarily imply causation (Gomez & Gomez, 1984).

Tocopherol prevents the oxidation of unsaturated fatty acids in cell membrane, so, maintaining their structure (Daintith, 1996). Therefore, it seems reasonable to speculate that the increases in tocopherol levels in infected leaf tissues may enhance flax resistance to PM by protecting cell membrane from oxidative damage associated with infection.

It is well known that the fungitoxic effect of most phenolics is attributed mainly to their interaction with lipids or phospholipids, causing an increase in fungal membrane permeability, leakage of cell contents, and cytoplasm aggregation (Dallagnol et al., 2011). Thus, increases in the levels of post-infectional phenolic compounds in leaf tissues may enhance flax resistance against infection by PM. The negative association between phenolics and susceptibility to PM, as we have demonstrated herein, has been supported by findings of Gawande et al. (2002), Avtar et al. (2003), Rao et al. (2007) and Satisha et al. (2008).

The MDA produced during lipid peroxidation is an indicator of cellular damage. It is well known that lipid peroxidation in the plasma membrane of cell wall by reactive oxygen species is caused by the occurrence of any type of stress to the cells (Dallagnol et al., 2011). In our study, MDA was the only component, which showed positive correlation with PM severity. This result may suggest that the post-infectional lipid peroxidation plays an important role in determining susceptibility of flax to PM. The high levels of post-infectional lipid peroxidation, as indicated by the enhanced production of MDA, in highly susceptible cultivars, could cause an increase in flax membrane permeability that, in turn, may lead to more disease severity.

Many reports suggest that the antioxidant ascorbic acid is involved in suppression of defense genes. For example, ascorbic acid reduced the elicitor-inducible phenolic synthesis in rice (Velazhahan &Vidhyasekaran, 1999) and tomato cells (Vera-Estrella et al., 1993). Ascorbic acid inhibited the oxidation of phenolics in epicotyls of *Vigna angularis* (Takahama, 1993) and spinach leaves (Takahama & Oniki, 1992). It also inhibited the oxidation of coniferyl alcohol by peroxidase leads to synthesis of lignin, another important defense-related compound (Ye et al., 1990). Pastori et al. (2003) showed that many defense genes, particularly those that encode pathogenesis-related proteins were activated in *Arabidopsis* mutant deficient in biosynthesis of ascorbic acid. In this study, a negative correlation was observed between ascorbic acid content and PM severity. This finding may suggest that ascorbic acid is involved in activation of defense genes. However, no conclusive biological explanation is available for the discrepancy between our finding and those reported by the others.

Our results are in agreement with Ashry & Mohamed (2012) who found that total soluble protein content in flax leaves infected with *Odium lini* Skoric increased significantly in resistant lines when compared with resistant parent but decreased significantly in susceptible lines when compared with resistant parent. In susceptible cultivars, powdery mildew fungi reduce plant protein because they take up amino acids from plants in order to grow biographically. So, plants cannot build up proteins properly. In contrast, the contribution of proteins in plant resistance to fungal infection is well documented in literature (Strange, 2003). We assessed proteins in infected flax leaves after the appearance of early disease symptoms. Therefore, it seems likely that the assessed proteins included both constitutive proteins and pathogenesis-related proteins, which are induced in response to pathogen attack. Constitutive protein has a role to play in plant defense through a variety of mechanisms. For instance, they may affect structural components of pathogen wall or interfere with synthesis of pathogen wall. They may also destabilize fungal membrane (Strange, 2003). On the other hand, pathogenesis-related proteins show strong antifungal activity. For example, some of them inhibit spore release and germination whereas others are associated with strengthening of the host cell wall and its outgrowths and papillae (Agrios, 2005).

The importance of peroxidase in disease resistance stems from its property to oxidize phenolic compounds to quinones and semiquinones, which are often more toxic to pathogens than the original phenols. Peroxidase not only oxidizes phenolics but also increases the rate of polymerization of such compounds into lignin-like substances, which are deposited in cell walls and papillae and interfere with the growth and development of the pathogen (Agrios, 2005). Direct antifungal effects of peroxidase on spore germination and mycelial growth have also been demonstrated (Joseph et al., 1998). Thus, it was reasonable to find a significant and negative correlation between peroxidase activity and PM severity.

Polyphenoloxidase (PPO) is an enzyme of broad distribution among plants. Most of the reports on PPO indicate a function to defend plants against pathogens. The mode of action proposed for PPO is based on its capacity to catalyze the hydroxylation of monophenols to diphenols and their oxidation to diquinones. The quinones formed may act in several ways leading to protection of plants (Melo et al., 2006). Thus, PPO showed a significant and negative correlation with PM severity. On the other hand, the correlation between phenolics and PM severity ($r = -0.845$, $P < 0.01$) was greater than that between PPO and PM severity ($r = -0.801$, $P < 0.01$)

These results suggest that flax resistance to PM may be related to the oxidative potential of phenolic composition of leaves rather than simply to a higher PPO activity (Melo et al., 2006).

Our results are in agreement with other reports, which showed a negative association between post-infectional activity of both peroxidase and PPO and susceptibility to PM in some hosts like garden pea (Kalia, 1998), mungbean

(Gawande et al., 2002), fenugreek (Avtar et al., 2003), and grapes (Roa et al., 2007).

Phenols, MDA and proteins showed the highest correlation with PM severity. In practical terms, the high r values mean that a primary selection to eliminate susceptible genotypes can be made relatively at early stage of growth after the appearance of the first visible symptoms. In this primary selection, only genotypes with high levels of phenols or low levels of MDA would be retained for further evaluation under field conditions or in the greenhouse. This could decrease the time and effort necessary for the development of resistant genotypes in breeding programs. However, this conclusion needs to be critically tested by evaluating more comprehensive samples and cultivars.

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