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ASCORBIC ACID OF SEEDS AND PROTEINS OF LEAVES AS BIOCHEMICAL MARKERS FOR RESISTANCE OF FLAX TO POWDERY MILDEW DISEASE

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ABSTRACT

A field trial was conducted in 2009/2010 and 2010/2011 growing seasons at Giza Agricultural Research Station to evaluate powdery mildew (PM) severity on 15 flax cultivars. In general, the tested cultivars could be divided into six distinct groups, i.e., highly susceptible (Corland and C.I. 2008), susceptible (Sofie and Marylin), moderately susceptible (Giza 8, Sakha 1, Giza 7, and Marshall), moderately resistant (Cass and Clay), resistant (Koto, Dakota, Wilden, and Bombay), and highly resistant (Ottawa 770B). The cultivars showed considerable variation in PM severity ranged from 8.05 on Ottawa 770 B to 97.02% on Cortland. Total free amino acids, total soluble proteins, total phenols, antioxidant enzymes (peroxidase and polyphenoloxidase), ascorbic acid, tocopherol, and malondialdehyde (MDA), as indicator of lipid peroxidation, were determined in uninfected seeds and in uninfected leaves of the tested cultivars. Pearson's correlation coefficient was calculated to measure the degree of association between PM severity and each component in linseeds or in leaves. All components, except free amino acids in linseeds and MDA in leaves, showed significant ($P<0.05$) or highly significant ($P<0.01$) negative correlation with PM severity. Free amino acids in linseeds were not correlated with PM severity, while MDA in leaves was positively correlated ($P<0.01$). Data for PM severity and level or activity of each component were entered into a computerized stepwise multiple regression analysis. Using the predictors supplied by stepwise regression, two one-factor models were constructed to predict PM severity. These models showed that PM severity differences were due largely to ascorbic acid of seeds and proteins of leaves, which accounted for 58.46 and 77.15%, respectively of the total variation in PM severity. The results of the present study suggest that ascorbic acid in uninfected seeds or total proteins in uninfected leaves can be used as biochemical markers to predict PM resistance in flax.

Key words: *Linum usitatissimum*, *Oidium lini*, resistance, biomarkers.

INTRODUCTION

Flax (*Linum usitatissimum* L.) is considered the most important fibre crop in Egypt; it ranks second after cotton (seedy fibre) regarding economic importance and production.

Powdery mildew (PM), caused by *Oidium lini* Škoric, is currently the most common, conspicuous, widespread, and easily recognized foliar disease of flax in Egypt. 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). In India, Pandy and Misra (1993) reported that as the disease increased, yield losses increased ranging from 11.8 to 38.9%; yield losses were larger when the disease appeared earlier in the season. Accurate assessment of losses due to the disease in Egypt has not been reported. However, Aly et al. (1994) found significant negative correlations between disease intensity ratings and agronomic traits (yield and yield components).

Currently, resistance is not available in flax cultivars commercially grown in Egypt (Aly at 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 the disease control (Aly et al., 1994).

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However, complete dependence on fungicides for the disease control carries risks for the producers, in that accurate coverage and distribution of fungicides may not be achieved and there are potential problems with correct timing of applications. Furthermore, increasing concern for the environment will likely mean stricter regulation of fungicide usage (Pearce et al., 1996).

Use of cultivars with PM resistance can resolve all these problems. However, successful screening for PM resistance in flax requires the development of a reliable method for quantification of resistance.

It has been suggested that a variety of substances contained in plant cells are involved in resistance or susceptibility to infection by pathogens. Among these, amino acids (Van Andel, 1966), proteins (Strange, 2003), phenols (Agrios, 2005), ascorbic acid (Vidyasekaran, 2008), tocopherol (Castle and Day, 1984), peroxidase (Agrios, 2005), polyphenoloxidase (Agrios, 2005), and malondialdehyde as indicator of lipid peroxidation (Göbel et al., 2003), have been reported.

However, from practical standpoint, apart from peroxidase (Reuveni et al., 1992) and amino acids (Aly et al., 2010), no attempts have been made to utilize these substances, in uninfected genotypes, as biochemical markers to predict resistance to diseases in breeding programs.

Therefore, the objectives of the present study were to (1) evaluate the relationship of each of these substances in uninfected seeds or leaves to PM severity ratings on flax and (2) develop statistical models to predict PM severity ratings by using these substances as biochemical predictors.

MATERIAL AND METHODS

Reactions of flax cultivars to PM

A field trial was conducted in 2009/2010 and 2010/2011 growing seasons at Giza Agricultural Research Station to evaluate PM severity on 15 flax cultivars. The experiment consisted of a randomised complete block design of three replications (blocks). Plots were 2x3 (6 m²) and 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. Disease severity was rated visually in the last week of April (Nutter et al., 1991).

Chemical analysis

Random samples of seeds, taken from the same seeds used in planting the field trial, were used for the chemical analysis. Leaves were used for the chemical analysis when plants were 60 days old. Biochemical components were determined as follows:

1. Total phenols

Levels of soluble phenols in fresh samples were determined in accordance with Dihazi et al. (2003). The absorbance of the developed blue colour was read at 725 nm. Gallic acid was used as standard and the amount of soluble phenols was expressed as mg gallic acid/100 g fresh weight.

2. Antioxidant enzymes

Peroxidase (EC 1.11.1.7) and polyphenoloxidase (EC 1.14.18.1) were assayed according to the method described by Kar and Mishra (1976). The colour intensity was read at 430 nm, and the enzyme activity was expressed as enzyme activity/gram fresh weight/hour.

3. Total soluble protein

The total soluble protein content in supernatant was determined according to Lowery et al. (1951) and was measured at 750 nm using spectrophotometer. The quantity of total soluble protein was calculated according to the standard curve of bovine albumin and expressed as mg/g fresh weight.

4. Ascorbic acid

Content of ascorbic acid (AsA) was estimated according to Mukherjee and Choudhuri (1983). The absorbance was recorded at 530 nm. The content of ascorbic acid was calculated from a standard curve plotted with known content of AsA and expressed as μg/g fresh weight.

5. Tocopherol

The absorbance of α-tocopherol was read at 520 nm against ethanol as a blank (Philip *et al.*, 1954). The content of α-tocophenol in the

extracts was calculated from the regression equation of the standard curve. The results were expressed in µg/g fresh weight.

6. Lipid peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content (a product of lipid peroxidation) according to the method of Heath and Packer (1968). MDA reacted with thiobarbituric acid (TBA) to form MDA-TBA. The amount of MDA-TBA complex was calculated from the extinction coefficient 155 ($mM^{-1}cm^{-1}$).

7. Total free amino acids

Total free amino acids were extracted with 70% ethanol and estimated by the method described by Moore and Stein (1954) using Ninhydrin reagent and expressed as mg/g fresh weight.

Statistical analysis

The experimental design of the field trials and the laboratory tests was randomised 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. Stepwise regression technique with the greatest increase in R^2 as the decision criterion was used to describe the effects of biochemical components on PM severity. Correlation and regression analysis was performed with a computerized program (SPSS Version 13).

RESULTS

Environmental conditions in 2009/2010 and 2010/2011 growing seasons were favourable for epiphytotic spread of the disease. This was apparent as these environmental conditions resulted in 97.02% PM severity on cultivar Cortland, which is known as highly susceptible (A.A. Aly, *personal observations*). In general, the tested cultivars could be divided into six distinct groups, i.e. highly susceptible (Cortland and C.I. 2008), susceptible (Sofie and Marylin), moderately susceptible (Giza 8, Sakha 1, Giza 7, and Marshall), moderately resistant (Cass and Clay), resistant (Koto, Dakota, Wilden and Bombay), and highly resistant (Ottawa 770 B). The cultivars showed considerable variation in PM severity, which ranged from 8.05 on Ottawa 770 B to 97.02% on Cortland (Table 1).

Table 1. Powdery mildew severity ratings on 15 flax cultivars and their disease categories under field conditions in Giza in 2009/2010 and 2010/2011 growing seasons

Cultivar	Disease severity [a]	Disease category [b]
Cortland	97.02 A	HS
C.I. 2008	95.86 A	HS
Sofie	89.61 B	S
Marylin	84.93 BC	S
Giza 8	77.52 CD	MS
Sakha 1	73.65 DE	MS
Giza 7	70.36 DE	MS
Marshall	64.72 E	MS
Cass	48.51 F	MR
Clay	39.03 F	MR
Koto	24.00 G	R
Dakota	21.67 G	R
Wilden	18.29 G	R
Bombay	17.68 G	R
Ottawa 770B	8.05 H	HR

[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 angle before carrying out the analysis of variance to normalize data and stabilize variance throughout the data range. Means followed by the same letter(s) 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 resistance (MR), resistant (R), and highly resistant (HR).

The level or activity of each component, in linseeds or in leaves, except MDA varied among cultivars (Tables 2 and 3). They can be determined rapidly and with small amounts of flax seeds or leaves; therefore, large number of genotypes can be tested without sacrificing significant amounts of seeds or plants.

Table 2. Determination of levels and activities of some biochemical components in uninfected seeds of 15 flax cultivars

Cultivar	Components					
	Free amino acids (mg/g fresh weight)	Total protein (mg/g fresh weight)	Ascorbic acid (µg/g fresh weight)	Tocopherol (µg/g fresh weight)	Peroxidase (activity/h/g fresh weight)	Polyphenoloxidase (activity/h/g fresh weight)
Ottawa 770B	15.35[a] B-D	15.46 B	42.52 A	23.11 A	4.16 BC	10.38 B
Dakota	13.10 CD	21.47 A	39.58 AB	12.36 C	8.00 A	19.84 A
Bombay	12.85 CD	14.87 B	32.32 A-C	18.15 B	4.56 B	10.35 B
Cass	17.57 B-D	14.52 BC	30.09 A-D	11.42 CD	3.01 EF	9.88 C
Koto	24.53 BC	11.81 C-E	26.27 B-D	8.02 E	2.65 EF	6.04 I
Clay	16.97 B-D	11.70 C-E	30.54 A-C	10.51 C-E	3.89 CD	8.02 E
Wilden	17.77 B-D	14.22 BC	30.72 A-C	10.15 C-E	3.88 CD	8.76 D
Marshall	19.33 B-D	12.71 B-D	28.12 B-D	9.67 C-E	3.21 DE	7.58 F
Cortland	42.18 A	9.50 E	16.71 D	8.94 DE	1.83 G	5.54 J
C.I. 2008	26.08 B	10.88 DE	26.40 B-D	7.47 E	1.77 G	4.72 K
Giza 7	17.97 B-D	13.24 B-D	28.16 B-D	9.51 C-E	2.98 EF	6.38 H
Giza 8	22.85 BC	13.69 BC	26.32 B-D	9.83 C-E	2.34 FG	7.56 F
Sakha 1	26.93 B	12.65 B-D	22.10 CD	7.51 E	2.88 EF	6.80 G
Marylin	8.03 D	9.56 E	26.52 B-D	7.88 E	1.75 G	3.65 L
Sofie	9.87 D	10.52 DE	26.62 B-D	10.04 C-E	3.68 CD	5.46 J

[a] 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.

Table 3. Determination of levels and activities of some biochemical components in uninfected leaves of nine flax cultivars

Cultivar	Components						
	Total protein (mg/g fresh weight)	Phenols (mg/g fresh weight)	Ascorbic acid (µg/g fresh weight)	Tocopherol (µg/g fresh weight)	Malondialdehyde (MDA) (n mol/g/ fresh weight)	Peroxidase (activity/h/g fresh weight)	Polyphenoloxidase (activity/h/g fresh weight)
Ottawa 770B	62.22[a] A	125.30 A	18.54 AB	63.16 A	10.65 A	32.77 AB	17.02 A
Dakota	61.05 AB	128.40 A	19.72 A	57.69 AB	13.36 A	37.68 A	17.68 A
Cass	57.96 A-C	109.90 AB	14.59 A-E	53.93 BC	13.16 A	31.05 A-C	12.52 BC
Wilden	54.90 B-D	93.53 A-C	12.55 C-E	50.27 CD	13.93 A	27.10 A-E	9.50 CD
Koto	53.78 CD	74.26 C-E	9.55 E-G	44.24 D-F	14.97 A	25.37 A-E	9.19 CD
Marshall	47.43 E	89.39 B-D	11.67 D-F	41.45 E-G	16.37 A	24.75 B-E	9.30 CD
Giza 7	50.16 DE	72.96 C-E	9.41 E-G	47.76 C-E	15.42 A	26.07 A-E	9.43 CD
Cortland	46.83 E	42.86 E	6.46 G	31.24 H-K	17.81 A	18.36 C-E	7.36 DE
C.I. 2008	44.07 E	54.66 DE	7.42 FG	34.24 G-I	17.29 A	19.91 C-E	7.64 DE

[a] 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 60 days old.

Pearson's correlation coefficient was calculated to measure the degree of association between PM severity and each component in linseeds (Table 4) or in leaves (Table 5). All components, except free amino acids in linseeds (Table 4) and MDA in leaves (Table 5), showed significant (P<0.05) or highly significant (P<0.01) negative correlation with PM severity. Free amino acids in linseeds were not correlated with PM severity, while MDA in leaves was positively correlated (P< 0.01).

Table 4. Relationship of levels and activities of some biochemical components in uninfected seeds of 15 flax cultivars and powdery mildew severity ratings on these cultivars

Component	r[a]
Free amino acids	0.378
Total protein	-0.654[b]**
Ascorbic acid	-0.765**
Tocopherol	-0.654**
Peroxidase	-0.654**
Polyphenoloxidase	-0.642**

[a] Pearson's correlation coefficient, which measures the degree of association between the designated component and powdery mildew severity rating
[b] Significant at P<0.01 (**)

Table 5. Relationship of levels and activities of some biochemical components in uninfected leaves of nine flax cultivars and powdery mildew severity ratings on these cultivars

Component	r[a]
Total protein	-0.878[b]**
Phenols	-0.804**
Ascorbic acid	-0.775*
Tocopherol	-0.847**
MDA	0.868**
Peroxidase	-0.783*
Polyphenoloxidase	-0.698*

[a] Pearson's correlation coefficient, which measures the degree of association between the designated component and powdery mildew severity rating
[b] Significant at P<0.05 (*) or P<0.01 (**)

Using the predictors supplied by stepwise regression, two one-factor models were constructed to predict PM severity. These models showed that PM severity differences were due largely to ascorbic acid of seeds (Figure 1) and proteins of leaves (Figure 2), which accounted for 58.46 and 77.15%, respectively of the total variation in PM severity. Stepwise regression also showed that levels or activities of other components did not make a statistically significant contribution to R^2 values of the generated models. Therefore, they were not included in these models.

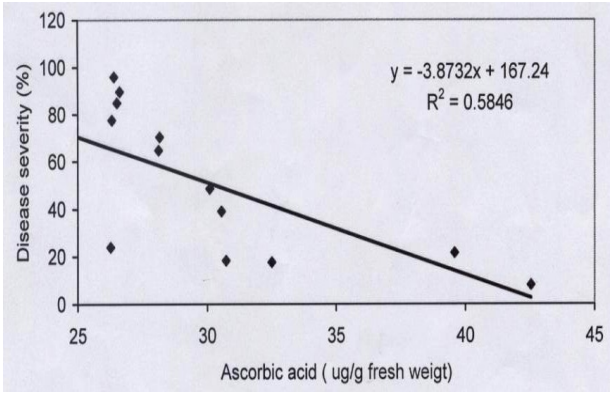

Figure 1. Effect of ascorbic acid content of uninfected flax seeds on powdery mildew severity

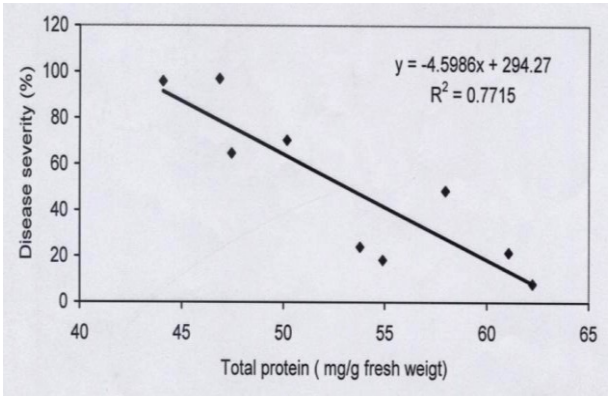

Figure 2. Effect of total protein content of uninfected flax leaves on powdery mildew severity

DISCUSSION

The conventional methods for evaluating flax genotypes for PM resistance are to test them under field or greenhouse conditions. Experience with flax PM showed that each method has its potential limitations. Under field conditions, susceptibility of genotypes to PM may be obscured by the nonhomogeneous distribution of the natural inoculum. In some years, susceptible genotypes may escape from infection due to the lack of natural inoculum or unfavourable environmental conditions. In addition, field tests are expensive and time-consuming. Admittedly, screening of genotypes under greenhouse conditions may overcome these difficulties and improve the efficiency of screening process; however, the greenhouse should be equipped with efficient and expensive air-conditioning system to maintain greenhouse temperature at about 25°C.

Thus, a new method should be developed to evaluate resistance of flax genotypes. This method should meet two requirements: it should be independent of the pathogen, and should reflect the genetic differences among genotypes. The biochemical components of linseeds or leaves (Tables 2 and 3) may meet these requirements for several reasons. The involvement of these components in resistance or susceptibility is well documented in the literature, as previously mentioned in the introduction.

It is well known that the type and degree of association between characters may facilitate or complicate the selection process in breeding programs. Selection for a character may result in an improvement or deterioration in other characters according to the type and degree of correlation. Hence, it was desirable to assess the type and degree of association between PM severity and each component in linseeds (Table 4) or in leaves (Table 5).

Data for PM severity and level or activity of each component were entered into a computerized stepwise multiple regression analysis. The analysis constructed a predictive model by adding predictors, in this case, levels and activities of the components, to the model in order of their contribution to R^2. The analysis was effective in eliminating those variables with little or no predictive value by incorporating into the model only those variables that made a statistically significant contribution to the R^2 value of the model (Podleckis et al., 1984).

In the present study, associations between PM severity and each component determined in uninfected seeds or in uninfected leaves were identified and the relative strength of these associations was measured by calculating Pearson's correlation coefficient (r). However, one should keep in mind that the significant r value should be interpreted with caution (Gomez and Gomez, 1984) because significant correlation does not necessarily imply causation. In other words, the results of the present study suggest that ascorbic acid in uninfected seeds or total proteins in uninfected leaves, which may or may not be parts of the PM resistance mechanisms, can be used as biochemical markers to predict PM resistance.

CONCLUSIONS

In practical terms, our results mean that a primary selection to eliminate susceptible genotypes can be made at early stage before planting (in case of using ascorbic acid of seed) or after planting (in case of using proteins of leaf). In this primary selection, only genotypes with high levels of ascorbic acid in seeds or with high levels of proteins in leaves would be retained for further evaluation under field conditions or in the greenhouse and thereby decrease the time and effort necessary for the development of resistant genotypes in breeding programs.

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