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USE OF PROTEIN ELECTROPHORESIS TO QUANTIFY RESISTANCE OF FLAX CULTIVARS TO RUST DISEASE

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ABSTRACT

Six flax cultivars were evaluated for rust resistance under field conditions in 2001/2002 and 2002/2003 growing seasons. The tested cultivars could be divided into two distinct groups. The first group included the susceptible cultivars Dakota, Wilden, Williston Brown, and Cortland, while the second group included the resistant cultivars Linore and C.I. 2008. There was a significant difference ($p \leq 0.05$) between any cultivar belonged to the first group and any cultivar belonged to the second group. However, the differences were nonsignifigant within each group. Proteins of the cultivars were separated by SDS-PAGE, and the obtained banding patterns were visualized by using the Coomassie-blue staining system. Data for rust ratings and amounts of protein fractions were entered into a computerized stepwise multiple regression analysis. Using the predictors supplied by stepwise regression, a four-factor model was constructed to predict rust severity. This model showed the rust severity differences were due largely to the protein fractions nos. 1, 18, 15 and 8, which accounted for 99.71% of the variation in severity ratings. These results indicate that SDS-PAGE of proteins may provide a supplementary assay to greenhouse and field tests to distinguish quantitatively between rust resistant or susceptible genotypes.

INTRODUCTION

Melampsora lini (Ehrenb.) Desmaz., is the causal agent of rust on cultivated flax, *Linum usitatissimum* L., this disease causes serious losses in the major flax-producing regions of the world including Australia, New Zealand, Egypt and the USA. The life cycle of flax rust pathogen is termed macrocyclic since it consists of all five possible spore stages, namely basidiospore, pycniospore, aeciospore, urediniospore, and teliospore. It is also referred to as autoecious since all spore stages occur on one hosts, asopposed to heteroecious where more than one host are involved. The urediniospore stage is a repeating stage and the on einvolved in spread of the disease from plant to plant (Coffey, 1983). So far, the uredinispore and teliospore have been the only stages encounted in Egypt.

This disease has served as a model for developing the gene-for-gene relatiuonship, which states that for each gene that conditions reaction in the host there is a corresponding gene in the parasite that conditions pathogenicity (Flor, 1971).

In Egypt, flax is grown for both seeds and fibers in the Nile Delta, in particular the northern governorates, This area is characterized by the prevalence of cool, wet weather during flax growing season. Such weather favors epiphytotic spread of the disease when virulent races occurs. The development of cultivars containing genes for vertical resistance has been one of the most successful methods for controlling flax rust. However, the

chief cause of impermanence of rust resistance in flax has been the appearance and rapid distribution of races of *M. lini* capable of attacking previously resistant cultivars (Statler, 1979). Thus, successful breeding for rust resisatnce in flax requires the development of a reliable method for quantifying resistance of flax genotypes to rust.

The use of gel electrophoresis to analyze plant protein and hence distinguisnh between and identify cultivars of crop species is a firmly established technique (Cook, 1988). Proteins are primaly products of gene expression and reflect gene system specificity in the best manner. Therefore, they are used as very effective markers for genotype identification and evaluation of the species and cultivar constitution (Konarev, 1988).

Some attempts were made to differentiate among flax cultivars by using protein electrophoresis. For eaxmple, Khalil (1981) found very high degree of similarity among electrohporetic protein banding patterns of resistance and susceptible cultivars to *M. lini*. Following infection, certain changes occurred in the protein patterns of the susceptible cultivar, but not in the that of the resistant one. The changes were in the form of a shift in the intensity of sone bands and the disappearance of some other bands. Such changes were not evident in the resistant cultivar (Bombay), probably due to the very limited activity of the fungus in that cultivar.

Lapina and Rullin (1985) analyzed the protein fractions electrophoretically in the stems of four flax varieties at different phases of growth. They reported that some fractions were present in each variety througout the growth period, and the greater number of fractions were found at the phase of rapid growth. They identified each variety with a characteristic protein fraction (or a group of fractions) at each stage of growth.

In a study of protein banding patterns of eight flax varieties differing in resistance to lodging and fungal diseases. Lapina (1989) reported that these patterns contained 15-22 bands, with the fewest being found in the patterns of the varieties susceptible or only moderately resistant to lodging and fungi. There were cultivar specific bands by which the cultivars could be identified.

Lapina and Kel'ner (1990) examined the electrophoretic characteristics of the seed protein of four flax cultivars differing in yield, resistance to lodging and resistance to fungal diseases. They found that there were differences between protein banding patterns of the studied cultivars, and that each pattern had bands in common and cultivar specific bands. There were 45 bands common to all the cultivars and 2-6 associated with the genotype of the particular seeds. They also reported that the cultivar, which had the widest range of economically useful traits had the highest number of bands in its pattern (71 bands).

Abd El-Salam (1998) differentiated by protein electrophoresis among six monogenic flax cultivars carrying the major genes for rust resistance. However, grouping the cultivars by cluster analysis based on their protein banding patterns was not related to their resistance to rust.

In the present study, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to develop a model to predict rust resistance in selected flax genotypes.

MATERIALS AND METHODS

Evaluation of flax genotypes for rust resistance

Experiments were conducted at Sakha Agricultural Research Station in 2001/2002 and 2002/2003 growing seasons. Experiment consisted of a randomized complete block design of 4 replicates (blocks). Plots were 2 x 3 m (6 m^2) and consisted of ten rows spaced 20 cm apart. Seeds of each genotype were sown by hand at a rate of 70 g/plot. Planting dates were in the first week of December. Disease severity was rated visually in the last week of April (Nutter *et al.*, 1991).

Extraction of proteins from flax seeds:

Protein extract was prepared according to Hussein (1992) in the following way: Seeds of healthy plants of flax cultivars C.I. 2008, Linore, Cortland, Williston Brown, Wilden and Dakota were slightly ground and defatted by diethyl either or chloroform for 4 to 5 days. After drying at room temperature, ground seeds were suspended in a solution (1-3 ml/g seeds) consisting of 12.5 g glucose and 1 g ascorbic acid dissolved in 100 ml phosphate buffer 8.3 and ground in liquid nitrogen to a fine powder.After thawing, the powder suspended in buffer was centrifugated at 19,000 rpm for 30 minurtes at 0°C. The protein content in the supernatant was adjusted to a concentration of 3 to 4 mg/ml according to Bradford (1976) spectrophotometric method by using bovine serum albumin as a standard protein.

Electrophoresis of dissociated protein (SDS-PAGE):

For electrophoresis of dissociated protein, each supernatant was mixed with an equal volume of a solution consisted of (by volume) 64% buffer (0.15 M Tris-HCL, pH 6.8); 20% glycerol; 6% sodium dodecyl sulfate (SDS); 10% 2-6 mercaptoethanol; and 0.1% bromophenol blue, before boiling in water bath for 3 minutes. Twenty-microliter samples (40 ug of proteins) were subjected to electrophoresis in 15% polyacrylamide gel prepared in 0.1% SDS (Laemmli, 1970 and Latorre *et al.*, 1995) and stained with Brilliant Blue R-250 (Weeke, 1973).

Statistical analysis

The experimental design of the field trials was a randomized complete block with four replicates (blocks). Analysis of varaince (ANOVA) of the data was performed with the MSTAT-C statistical package (A microcomputer Program for the Design, Management and Analysis of Agronomic Research Experiments, Michigan State Univ., USA). Duncan;s multiple range test was used to separate genotypes into susceptible and resistant classes.

Gel was scanned for band R_f (position) and amount (%) by the gel documentation system AAB (Advanced American Biotechnology 1166). Data were analyzed by a stepwise multiple regression using a computerized program. In this analysis, rust severity (dependent variable) was predicted by using the amounts of proteins as predictors (independent variables).

RESULTS and DISCUSSION

Evaluation of flax cultivars for rust severity (Table 1) revealed that the tested cultivars could be divided into two distinct groups. The first group

included the susceptible cultivars Dakota, Wilden, Williston Brown, and Cortland, while the second group included the resistant cultivars Linore and C.I. 2008. There was a significant difference between any cultivar belonged to the first group and any cultivar belonged to the second group. However, the differences were nonsignificant within each group.

Table (1): Reaction of six flax cultivars to rust under field conditions in Sakha in 2001/2002 and 2002/2003 growing seasons.

Cultivar	Rust severity [a] %
Dakota	4.05a
Wilden	6.00a
Williston Brown	5.11a
Cortland	5.48a
Linore	1.00b
C.I. 2008	0.73b

[a]Rust severity was the number of uredinia/plant in a random sample of 10 plants/plot. Each value was the mean of the two growing seasons. Means followed by the same letter were not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

A total of 44 protein bands were identified among the 6 cultivars that were analyzed (Fig. 1 and Table 2).

Table (2): Protein banding patterns for flax cultivars obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250.

Band no.	Mobility	Flax cultivar					
		Dakota	Wilden	Williston Brown	Cortland	Linore	CI 2008
1	3	1.50[a]	0.52	1.98	0.68	0.00	0.00
2	4	0.00	0.00	0.00	0.00	0.61	0.00
3	7	0.00	0.00	0.00	0.00	0.00	0.67
4	18	0.00	0.00	0.00	0.00	1.15	2.64
5	19	0.00	0.00	0.00	0.19	0.00	0.00
6	20	0.00	0.73	0.52	0.00	0.00	0.00
7	21	0.86	0.00	0.00	0.00	0.00	0.00
8	27	0.48	0.33	0.34	0.61	0.00	1.40
9	30	0.00	0.00	0.00	0.00	1.69	0.00
10	32	0.00	0.00	0.00	0.58	0.00	0.00
11	33	0.66	0.58	0.46	0.00	0.00	0.00
12	41	1.91	0.00	0.00	1.55	1.71	3.97
13	42	0.00	1.76	1.61	0.00	0.00	0.00
14	46	4.09	2.23	1.83	2.95	4.16	4.41
15	52	0.00	0.00	2.05	1.87	0.00	0.00
16	53	0.00	1.12	0.00	0.00	0.00	0.00
17	60	0.00	0.00	0.00	0.67	0.65	0.66
18	61	0.00	0.47	0.50	0.00	0.00	0.00
19	74	0.00	0.00	6.04	0.00	0.00	0.00
20	78	0.00	0.00	13.10	0.00	0.00	0.00
21	79	0.00	18.43	0.00	19.16	0.00	0.00
22	80	0.00	0.00	0.00	0.00	22.01	0.00
23	81	0.00	0.00	0.00	0.00	0.00	19.60
24	90	2.49	0.00	0.00	0.00	0.76	0.00
25	91	0.00	0.00	0.00	1.39	0.00	0.66
26	92	0.00	0.00	2.67	0.00	0.00	0.00
27	105	0.00	0.00	15.90	0.00	0.00	0.00
28	106	0.00	15.30	0.00	14.12	0.00	0.00
29	108	0.00	0.00	0.00	0.00	0.00	12.82
30	109	0.00	0.00	0.00	0.00	13.91	0.00
31	122	21.46	0.00	27.25	0.00	0.00	0.00
32	124	0.00	24.00	0.00	0.00	0.00	0.00
33	125	0.00	0.00	0.00	21.06	0.00	0.00
34	127	0.00	0.00	0.00	0.00	20.60	19.55
35	137	0.00	0.00	0.00	0.00	2.18	0.00
36	140	0.00	0.00	0.00	1.76	0.00	0.00
37	141	0.00	0.00	0.00	0.00	0.00	0.84
38	142	0.00	0.48	0.00	0.00	0.00	0.00
39	156	0.00	0.00	4.08	6.73	0.00	0.00
40	157	0.00	6.02	0.00	0.00	6.38	7.78
41	169	27.75	0.00	21.68	0.00	0.00	0.00
42	171	0.00	0.00	0.00	25.66	0.00	0.00
43	172	0.00	26.83	0.00	0.00	0.00	0.00
44	173	0.00	0.00	0.00	0.00	24.18	25.00

[a] Amount (%) of the designated protein fraction.

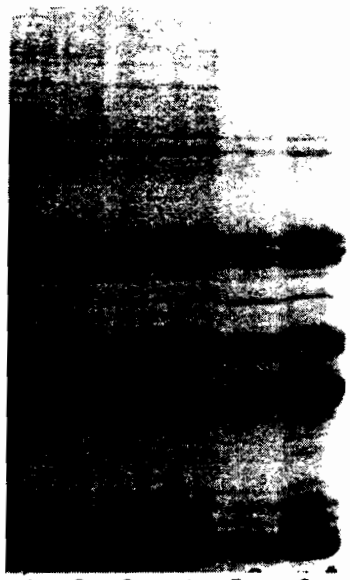

Fig. (1): Protein patterns obtained by SDS-PAGE from seeds of six flax culrivars. Flax cultivars were C.I. 2008 (1), Linore (2), Wilden (3), Cortland (4), Williston Brown (5), and Dakota (6).

No single cultivar was stained for all the 44 bands. Dakota showed the least number of bands (9 bands), while the other cultivars showed a number of bands ranged from 13 to 15. Band no. 14 was the only band, which was common to all the cultivars. Each cultivar was characterized by unique band(s). For eaxmple, band no. 7 was unique to Dakota. Wilden was characterized by the unique bands 16, 32, and 38.

Pearson correlation coefficient was calculated to measure the degree of association between rust severity and the amount (%) of each separated protein (Table 3). However, no single protein fraction was satisfactorily correlated with rust severity.

Table (3): Relationship of rust severity[a] on six flax cultivars and protein content[b] of seeds of these cultivars.

No.[c]	r[d]	No.	r	No.	r	No.	r
1	0.804	12	-0.415	23	-0.492	34	0.433
2	-0 414	13	-0.180	24	-0.366	35	-0.616
3	-0 545	14	-0.057	25	0.233	36	-0.366
4	-0 719	15	0.628	26	0.366	37	0.433
5	-0 366	16	0.526	27	0.526	38	-0.414
6	0 024	17	-0.250	28	0.366	39	0.617
7	-0.545	18	0.700	29	0.760	40	-0.700
8	-0 014	19	0.366	30	-0.414	41	0.366
9	-0 367	20	0.366	31	-0.726	42	0.433
0	-0 137	21	0.757	32	0.366	43	0.526
11	-0.312	22	-0.366	33	0.526	44	-0.712

[a] Mean of uredinia/plant in a random sample of 10 plants/plot.
[b] Amount of protein (%). [c] No. of protein fraction.
[d] Pearson corelation coefficient, which measures the degree of association between rust severity and the designated protein. Tabulated value was 0.811 (P = 0.05) or 0.729 (P = 0.10).

Data for rust ratings and amounts of protein fractions were entered into a computerized stepwise multiple regression analysis. The analysis constructed a predictive model by adding predictors, in this case amounts of protein fractions, 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 varaibles that made a satisfactory significant contribution to the R^2 value of the model (Podleckis *et al.*, 1984). Using the predictors supplied by stepwise regression, a four-factor model was constructed to predict rust severity (Table 4). This model showed that rust severity differences were due largely to the protein fractions nos. 1, 18, 15, and 8, which accounted for 99.71% of the variation in severity ratings.

Table (4): Regression equation that describes the effects of some protein fractions (X_5) on severity[a] of flax rust (Y).

Srepwise regression model	R^2 [b]	F-value
$Y = -0.205 + 2.124X_1 + 5.829X_{18} + 0.538X_{15} - 0.686X_8$	99.71	86.78*

[a] Rust severity was measured as the number of uredinia/plant in a random sample of 10 plants/plot.

[b] Coefficient of determination. Relative contributions of the predictors X_1, X_{18}, X_{15}, and X_8 to R^2 were 64.606, 29.852, 3.640, and 1.615%, respectively. F-value was significant at $P = 0.10$ (X).

DISCUSSION

The conventional methods for evaluating flax cultivars for rust resistance are to evaluate them under field and greenhouse conditions. Experience with flax rust showed that its method has its potential limitations. Under field conditions, susceptibility of cultivars to rust may be obscured by the nonhomogeneous distribution of the natural inoculum. In some years, susceptible cultivars may escape from infection due to the lack of inoculum or the prevailing of unfavourable environemntal 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 expensive cooling system to maintain greenhouse temperature at about 20°C. Thus, a new method should be developed to evaluate resistance of flax genotypes to rust. This method should meet two requirements. It should be independent of the pathogen, and should reflect the genetic differences among cultivars. SDS-PAGE of proteins reasonably meet these requirements for several reasons. Amino acid sequences of polypeptides (components of proteins) are dependent on nucleotide sequences of their coding genes; therefore, an analysis of protein variations among flax genotypes by electrophoresis, approximates an analysis of their genetic variation (Markert and Faulhaber, 1965). Electrophoretic patterns can be obtained rapidly and with small amounts of tissues. Therefore, large number of single plant selections can be tested without sacrificing the plants (Wheeler *et al.*, 1971).

The utility of the electrophoretic data depends on the method for statistical analysis. Multiple regression was a logical choice for construction of a predictive model, but the complex nature of banding patterns warranted a method to eliminate bands with no predictive value. Stepwise regression is the best variable selection procedure because it eliminates from the model any variable whose contribution to predictive ability is statistically insignificant (Draper and Smith, 1981 and Podleckis *et al.*, 1984).

In the present study, satisfactory visualizations of banding patterns were obtained by using the Coomassie blue staining system for general proteinns, and the stepwise regression model they generated proved effective in predicting rust severity from banding patterns. The model accounted for 99.71% of the explained (model) variation in rust severity.

The most common technique for selection of rust-resistant flax cultivars has been through ratings of visible foliar symptoms. The time and effort involved in these selection tests have limited plant breeders in selecting rust-resistant genotypes. SDS-PAGE of proteins such as that described here may provide a supplementary assay to greenhouse and field tests to distinguish between rust resistant or susceptible genotypes quantitatively.

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إستعمال التفريد الكهربى للبروتينات للتعبير الكمى عن مقاومة أصناف الكتان لمرض الصدأ

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قيمت ستة أصناف من الكتان وذلك من حيث المقاومة لمرض الصدأ تحت ظروف الحقل خلال موسمى ٢٠٠١/٢٠٠٢ و ٢٠٠٢/٢٠٠٣. انقسمت الأصناف إلى مجموعتين محددتين. المجموعة الأولى اشتملت على الأصناف القابلة للإصابة داكوتا و ولدن و ولستون براون و كورتلاند، فى حين اشتملت المجموعة الثانية على الصنفين المقاومين لينور والصنف ٢٠٠٨. كان الفرق فى شدة الإصابة معنويا بين أى صنف يقع فى المجموعة الأولى وأى صنف يقع فى المجموعة الثانية، فى حين كانت الفروق بين الأصناف داخل كل مجموعة غير معنوية. استعملت تقنية التفريد الكهربى لفصل بروتينات الأصناف وذلك بعد تفكيك هذه البروتينات باستعمال مادة صوديوم دوديسيل سلفيت. استعملت مادة الكوماسى بلو لصبغ أنماط البروتينات المتحصل عليها. أمكن باستخدام أسلوب الانحدار المتعدد المرحلى التوصل إلى نموذج رياضى لوصف العلاقة بين شدة المرض (متغير تابع) والبروتينات المفصولة (متغير مستقل). أظهر هذا النموذج أن ٩٩,٧١% من التباين فى شدة المرض من الممكن أن يعزى إلى تأثير البروتينات أرقام ١ و ١٨ و ١٥ و ٨. تدل نتائج الدراسة الحالية على أنه من الممكن استخدام تقنية التفريد الكهربى للبروتينات كوسيلة مكملة لاختبارات الصوبة والحقل للتفرقة الكمية بين تراكيب الكتان الوراثية المقاومة أو القابلة للإصابة بالصدأ.