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USE OF PROTEIN ELECTROPHORESIS TO QUANTIFY RESISTANCE OF FLAX GENOTYPES TO POWDERY MILDEW DISEASE

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ABSTRACT

Ten flax genotypes were evaluated for powdery mildew resistance under field conditions in 2002/2003 and 2003/2004 growing seasons. Lines 420/4, 421/43, and 421/60 showed the lowest ratings of disease severity, while line 110/3 showed the highest rating. The remaining genotypes showed intermediate ratings ranging from 40.93 to 57.89%. Proteins of cultivar seeds were separated by SDS-PAGE, and the obtained banding patterns were visualized by using the silver nitrate staining system. Data for powdery mildew ratings and amounts of protein fractions were entered into a computerized stepwise multiple regression. Using the predictors supplied by stepwise regression, a four-factor model was constructed to predict powdery mildew severity. This model showed that powdery mildew severity differences were due largely to the protein fractions no_s. 33, 31, 6, and 53, which accounted for 95.43% of the total variation in severity ratings. This result indicates that SDS-PAGE of seed proteins may provide a supplementary assay to field tests to distinguish quantitatively between powdery mildew resistant or susceptible genotypes.

INTRODUCTION

Powdery mildew (PM) of flax (*Linum usitatissimum* L.) is caused by the obligate parasite *Oidium lini* Škoric. This fungus is found on flax in Egypt only in its imperfect (conidial) stage. The pathogen infects all the aboveground flax organs including stems, leaves, flowers, and capsules. PM occurs annually in all flax production areas in Egypt (A.A. Aly, *personal observations*).
Significant negative correlations were found between disease intensity ratings and agronomic traits (Aly *et al.*, 1994). Currently, all commercially grown flax cultivars are susceptible to the disease, although field observations indicated that some experimental lines were more susceptible than others (Aly *et al.*, 2001).

Fungicides are currently the only commercially available management practices for controlling the disease and minimizing associated losses in seed and straw yield (Aly *et al.*, 1994 and Mansour, 1998). Complete dependence on fungicides for the disease control carries risks for the procedures, in that accurate coverage and distribution of fungicides may not be achieved and there are potential problems with correct timing of application. Furthermore, increasing concern for the environment will likely mean greater regulation of pesticide usage (Pearce *et al.*, 1996).

Use of cultivars with PM resistance can resolve all these problems. Currently, field evaluation is the only reliable method to distinguish flax genotypes with PM resistance. However, the precision of field evaluation of genetic resistance is adversely affected by environmental variation and heterogeneous levels of natural inoculum. In addition, field evaluation is expensive and time-consuming.

Therefore, another reliable method, either alternative or complementary to field evaluation, is required for identification of flax genotypes with PM resistance.

The use of gel electrophoresis to analyze plant protein and hence distinguish between and identify cultivars of crop species is a firmly established technique (Cook, 1988). Proteins are primary 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 example, Khalil (1981) found very high degree of similarity among electrophoreteic protein banding patterns of resistant and susceptible flax cultivars to *Melampsora lini*. Following infection, certain changes occurred in the protein patterns of susceptible cultivars, but not in that of the resistant one. The changes were in the form of a shift in the intensity of some 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 throughout the growth period, and that 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.

Flax cultivar specific protein bands, separated by electrophoresis, may be useful as biochemical markers for seed purity tests (Hussein *et al.*, 2002) or cultivar identification (El-Sweify *et al.*, 2003).

In the present study, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was employed to develop regression models to quantify PM resistance in selected flax genotypes. To the best of our knowledge, this approach has not been employed previously in the quantification of flax resistance to PM.

MATERIALS AND METHODS

Evaluation of flax genotypes for PM resistance:

Experiments were conducted over two successive growing seasons on a heavy clay soil at Sakha Agricultural Research Station, beginning in the fall of 2002. Experiments consisted of a randomized complete block design of five replications (blocks). Plots were 4.5 m^2 (1.5 x 3.0 m) and consisted of 21 rows spaced 7 cm a part. Plots were mechanically planted with flax genotypes at a rate of 50 kg/feddan on 15 November 2002 and 20 November 2003. Disease severity was rated visually in the last week of April each year. Disease severity was measured as the percentage of infected leaves/plant in a random sample of 10 plants/plot (Nutter *et al.*, 1991).

Extraction of proteins from flax seeds:

Protein extract was prepared according to Hussein (1992) in the following way: Seeds of genotypes 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 1g ascorbic acid dissolved in 100ml phosphate buffer, pH 8.3 and ground in liquid nitrogen to a fine powder. After thawing, the powder suspended in buffer was centrifuged at 19,000 rpm for 30 minutes 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 consisting of (by volume) 64% buffer (0.15 M Tris-HCl, pH 6.8); 20% glycerol; 6% SDS; 10% 2-6 mercaptoethanol, and 0.1% bromophenol blue, before boiling in a water bath for 3 minutes. Twenty-microliter samples (40 µg of protein) were subjected to electrophoresis in 5-20% gradient polyacrylamide prepared in 0.1% SDS (Laemmli, 1970). Electrophoresis was conducted at 10°C, for 4hr on 5-20% gradient polyacrylamide gel with 3.5% stacking gel, at 30 and 15 mA, respectively, until the dye band reached the bottom of the separating gel (Laemmli, 1970). Electrophoresis was preformed in a vertical slab mold (16 x 18 x 0.15 cm). Gel was stained with silver nitrate for the detection of protein bands (Sammons *et al.*, 1981).

Statistical analysis of the data:

The experimental design of the field trials was a randomized complete block with five replications.

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Analysis of variance (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 compare genotypes means. Percentage data were transformed into arc sine angles before carrying out ANOVA to produce approximately constant variance.

Gel was scanned for band R_f (position) and amount (%) by the gel documentation system AAB (Advanced American Biotechnology 1166). Stepwise regression technique with greatest increase in R^2 as the decision criterion was used to describe the effects of proteins (predictors or independent variables) on PM severity (dependent variable). Correlation and regression analyses were preformed with a computerized program.

RESULTS AND DISCUSSION

Lines 420/4, 421/43, and 421/60 showed the lowest ratings of disease severity, while line 110/3 showed the highest rating. The remaining genotypes showed intermediate ratings ranging from 40.93 to 57.89% (Table 1).

Table 1: Reaction of 10 flax genotypes to powdery mildew (PM) under field conditions in Sakha in 2002/2003 and 2003/2004 growing seasons.

Genotype	PM severity [a] (%)
Giza 7	47.39 [AB]
420/140	49.44 [AB]
420/4	30.93 [B]
420/153	57.82 [AB]
421/3	40.93 [AB]
421/43	36.58 [B]
421/60	35.79 [B]
110/3	67.46 [A]
282/37	57.89 [AB]
282/98	38.74 [AB]

[a] PM severity was the percentage of infected leaves/plant in a random sample of 10 plants/plot. Each value was the mean of two growing seasons. Means followed by the same letter(s) were not significantly different ($p < 0.05$) according to Duncan's multiple range test.

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 SDS-PAGE approximates an analysis of their genetic variation (Aly *et al.*, 2003). Electrophoretic patterns can also 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).

In the present study, a total of 60 protein bands were identified among the 10 genotypes that were analyzed (Fig. 1 and Table 2). This large number of bands was due to the effect of SDS, which dissociated each oligomeric protein into its subunits (Bohinski, 1983). When protein preparation was treated with mercaptoethanol and SDS, the mercaptoethanol disrupted (reduced) all disulfide (-S-S-) bonds present in proteins, whereas the detergent SDS bound to all regions of protein and unravelled all intramolecular protein associations. This resulted in total disruption of associated subunits organization and then yielded SDS-carrying, highly-anionic polypeptide chains (Clark and Switzer, 1977). The large number of the detectable bands could also be attributed to the use of silver nitrate. This salt is a highly sensitive visualization technique for detection of the small amounts of proteins, which cannot be seen with Coomassie

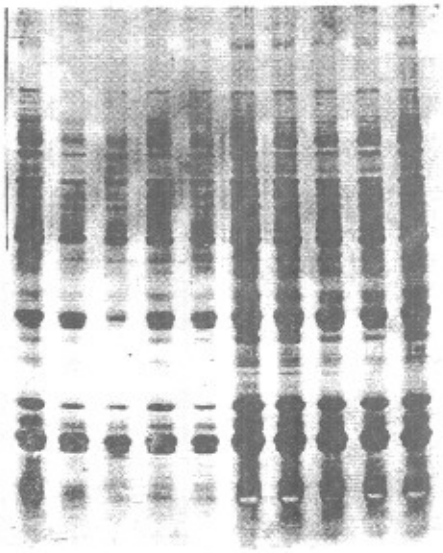

Fig. 1. Protein patterns obtained by SDS-PAGE (gradient gel) from seeds of ten flax genotypes. Genotypes in lanes 1 through 10 were (1) Giza 7, (2) 420/140, (3) 420/4, (4) 420/153, (5) 421/3, (6) 421/43, (7) 421/60, (8) 110/3, (9) 282/37 and (10) 282/98.

Brilliant Blue R-250 (CBB). Generally, silver nitrate is 100-fold more sensitive than CBB (Andrews, 1986). No single genotype was stained for all the 60 bands. Similarly, no single band was common to all the genotypes. Line 420/140 showed the least number of bands (8 bands), while the other genotypes showed a number of bands ranging from 10 to 15. Each genotype was characterized by unique bands. For example, bands no$_s$. 2, 20, 24, 29, 40, 44, 46, 50, and 60 were unique to Giza 7.

Table 2. Protein banding patterns for ten flax genotypes obtained by SDS-PAGE (gradient gel) and stained with silver nitrate.

Band		Genotype									
No.	Position	Giza 7	420/140	420/4	420/153	421/3	421/43	421/60	110/3	282/37	282/98
1	0	2.20 [a]	0.00	0.00	2.63	0.00	0.00	3.53	1.73	1.44	2.54
2	23	9.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	25	0.00	9.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	26	0.00	0.00	9.97	14.23	10.94	0.00	8.05	9.63	10.49	0.00
5	27	0.00	0.00	0.00	0.00	0.00	10.86	0.00	0.00	0.00	10.07
6	32	3.24	3.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	33	0.00	0.00	7.91	0.00	0.00	0.00	0.00	3.06	3.23	3.21
8	34	0.00	0.00	0.00	0.00	3.62	3.76	2.90	0.00	0.00	0.00
9	41	2.79	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.04
10	42	0.00	0.00	0.00	0.00	0.00	2.64	0.00	0.00	2.70	0.00
11	47	4.03	5.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.52
12	48	0.00	0.00	4.25	7.05	7.24	0.00	0.00	5.79	2.77	0.00
13	49	0.00	0.00	0.00	0.00	0.00	0.00	5.99	0.00	0.00	0.00
14	55	0.00	0.00	0.00	0.00	0.00	0.00	2.86	0.00	0.00	0.00
15	56	0.00	0.00	3.40	7.52	3.51	0.00	0.00	5.62	3.35	0.00
16	61	0.00	7.70	2.95	0.00	3.56	0.00	0.00	0.00	0.00	0.00
17	62	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.42	0.00
18	72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.59
19	74	0.00	0.00	0.00	0.00	0.00	19.43	13.35	0.00	0.00	0.00
20	75	16.54	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 2. Cont.

Band		Genotype									
No.	Position	Giza 7	420/140	420/4	420/153	421/3	421/43	421/60	110/3	282/37	282/98
21	77	0.00	22.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
22	78	0.00	0.00	18.23	9.37	0.00	0.00	0.00	10.48	0.00	0.00
23	79	0.00	0.00	0.00	0.00	10.38	0.00	0.00	0.00	8.58	0.00
24	85	9.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25	88	0.00	0.00	0.00	0.00	7.97	7.87	0.00	0.00	0.00	0.00
26	89	0.00	0.00	0.00	7.29	0.00	0.00	0.00	0 00	0.00	8.52
27	90	0.00	0.00	0.00	0.00	0.00	0.00	3.85	7.37	8.01	0.00
28	96	0.00	0.00	0 00	0.00	0.00	0.00	4.59	0.00	0.00	0.00
29	106	3.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	107	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.96
31	108	0.00	0.00	0.00	4.49	0.00	0.00	0.00	0.00	0.00	0.00
32	109	0.00	0.00	0.00	0.00	0.00	5.73	4.09	0.00	0.00	0.00
33	110	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.02	5.01	0.00
34	116	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.28
35	117	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.51	0.00	0.00
36	118	7.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.04	0.00
37	119	0.00	11.48	16.65	15.62	12.63	0.00	0.00	0.00	0.00	0.00
38	121	0.00	0.00	0.00	0.00	0.00	7.21	0.00	0.00	0.00	0.00
39	123	0.00	0.00	0.00	0.00	0.00	0.00	7.81	0.00	0.00	4.16
40	130	2.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 2. Cont.

Band		Genotype									
No.	Position	Giza 7	420/140	420/4	420/153	421/3	421/43	421/60	110/3	282/37	282/98
41	131	0.00	0.00	0.00	0.00	6.90	0.00	0.00	0.00	0.00	3.01
42	132	0.00	0.00	0.00	0.00	0.00	2.93	3.03	2.80	3.03	0.00
43	138	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.14
44	142	3.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
45	143	0.00	0.00	0.00	0.00	0.00	5.95	5.63	5.80	5.25	0.00
46	164	8.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
47	165	0.00	0.00	0.00	0.00	0.00	0.00	8.52	0.00	0.00	0.00
48	166	0.00	11.94	8.94	0.00	0.00	0.00	0.00	7.73	7.98	7.44
49	167	0.00	0.00	0.00	7.29	8.72	0.00	0.00	0.00	0.00	0.00
50	174	3.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
51	175	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.25
52	176	0.00	0.00	0.00	0.00	0.00	14.64	0.00	0.00	0.00	0.00
53	181	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.52	0.00
54	183	0.00	0.00	13.11	11.06	0.00	0.00	0.00	0.00	0.00	0.00
55	185	0.00	0.00	0.00	0.00	11.58	0.00	0.00	0.00	0.00	0.00
56	186	7.92	0.00	0.00	0.00	0.00	0.00	10.32	0.00	0.00	0.00
57	187	0.00	12.48	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
58	189	0.00	0.00	0.00	0.00	0.00	3.86	0.00	11.09	0.00	0.00
59	207	0.00	0.00	14.61	0.00	0.00	0.00	0.00	0.00	0.00	9.58
60	209	16.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

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

Table 3. Relationship between PM severity [a] on 10 flax genotypes and protein content [b] of seeds from these genotypes.

No. [c]	r [d]	No.	r	No.	r	No.	r
1	0.221	16	- 0.169	31	0.347	46	0.036
2	0.036	17	0.349	32	- 0.438	47	- 0.311
3	0.067	18	- 0.223	33	0.738 *	48	0.182
4	0.332	19	- 0.436	34	- 0.223	49	0.108
5	- 0.223	20	0.036	35	0.635 *	50	0.036
6	0.077	21	0.067	36	0.286	51	- 0.223
7	- 0.148	22	0.077	37	- 0.144	52	- 0.287
8	- 0.483	23	0.107	38	- 0.287	53	0.349
9	- 0.148	24	0.036	39	- 0.311	54	- 0.132
10	0.052	25	- 0.333	40	0.036	55	- 0.157
11	- 0.146	26	0.060	41	- 0.243	56	- 0.238
12	0.406	27	0.607 x	42	0.215	57	0.067
13	- 0.311	28	- 0.311	43	- 0.223	58	0.523
14	- 0.311	29	0.036	44	0.036	59	- 0.531
15	0.601 x	30	- 0.223	45	0.222	60	0.036

[a] Percentage of infected leaves/plant in a random sample of 10 plants/plot. [b] Amount of protein (%). [c] No. of protein fractions.
[d] Pearson correlation coefficient, which measured the degree of association between PM severity and the designated protein fraction, was significant at $p < 0.10$ (x) or $p < 0.05$ (*).

Table 4. Regression equation that describes the effects of some protein fractions (X_s) on severity[a] of flax powdery mildew.

Stepwise regression model	R^2 [b]	F-value[c]
Y = 36.60 + 6.15 **X33** + 4.73 **X31** + 3.54 **X6** – 0.90 **X53**	95.43%	26.08***

[a] PM severity was measured as the percentage of infected leaves/plant in a random sample of 10 plants/plot.
[b] Coefficient of determination. Relative contribution of the predicators X33, X31, X6, and X53 to R^2 were 54.44, 22.71, 14.65, and 3.63%, respectively.
[c] F. value is significant at $P < 0.005$ (***).

Pearson correlation coefficient was calculated to measure the degree of association between PM severity and the amounts of the separated protein fractions (Table 3). However, few proteins were satisfactory correlated with PM severity. Thus, of the 60 correlation coefficients shown in Table 3, only 4 (6.67 %) were significant ($p < 0.10$ or $p < 0.05$).

Data for PM severity and amounts of protein fractions were entered into a computerized stepwise multiple regression analysis. The analysis constructed predictive models by adding predictors, in this case, amounts of protein fractions, to the models 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 models only those variables that made a satisfactory 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 PM severity (Tables 4). This model showed that PM severity differences were due largely to the protein fractions nos. 33, 31, 6, and 53, which accounted for 95.43% of the total variation in severity ratings.

The utility of the electrophoretic data depends on the method of statistical analysis. Multiple regressions was a logical choice for construction of predictive models, 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 visualization of banding patterns were obtained by using the silver nitrate staining system for general proteins, and the stepwise regression model they generated proved effective in predicting PM severity from banding patterns. Therefore, SDS-PAGE of proteins, such as that described herein, may provide a supplementary assay to field trials to distinguish between PM resistant or susceptible genotypes quantitatively.

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إستعمال التفريد الكهربى للبروتينات للتعبير الكمى عن مقاومة التراكيب الوراثية للكتان لمرض البياض الدقيقى

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قيمت عشرة تراكيب وراثية من الكتان وذلك من حيث المقاومة لمرض البياض الدقيقى ، تحت ظروف الحقل ، خلال موسمى ٢٠٠٢/٢٠٠٣ و ٢٠٠٣/٢٠٠٤. أظهرت السلالات ٤/٤٢٠ و ٤٣/٤٢١ و ٦٠/٤٢١ أقل شدة إصابة فى حين أظهرت السلالة ٣/١١٠ أعلى شدة إصابة. أما باقى السلالات فقد أظهرت درجات متوسطة من شدة الإصابة تراوحت ما بين ٤٠,٩٣ إلى ٥٧,٨٩%. إستعملت تقنية التفريد الكهربى لفصل بروتينات بذرة الأصناف وذلك بعد تفكيك هذه البروتينات باستعمال مادة صوديوم دوديسيل سلفيت. إستعمل ملح نترات الفضة لصبغ أنماط البروتينات المتحصل عليها ، أمكن – باستخدام أسلوب الإنحدار المتعدد المرحلى – التوصل إلى نموذج رياضى لوصف العلاقة بين شدة المرض (متغير تابع) والبروتينات المفصولة (متغير مستقل). أظهر هذا النموذج أن ٩٥,٤٣% من التباين الكلى فى شدة المرض من الممكن أن يعزى إلى تأثير البروتينات أرقام ٣٣ و٣١ و٦ و٥٣. تدل نتيجة الدراسة الحالية على أنه من الممكن إستخدام تقنية التفريد الكهربى للبروتينات – كوسيلة مكملة لاختبارات الحقل – للتفرقة الكمية بين تراكيب الكتان الوراثية المقاومة أو القابلة للإصابة بمرض البياض الدقيقى.