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LACK OF RELATIONSHIP BETWEEN PROTEIN ELECTROPHORETIC PATTERNS OF FLAX GENOTYPES AND THEIR SUSCEPTIBILITY TO POWDERY MILDEW

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ABSTRACT

Proteins of ten flax genotypes having different levels of susceptibility to powdery mildew (PM) were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie Brillian Blue R-250 (CBB) or silver nitrate (SN). Cluster analsysis of the protein banding patterns by the unweighted pair-group method based on arithmetic means (UPGMA) placed the genotypes in several groups with overall similarity levels ranged from 66.56 for SN to 95.65% for CBB, which indicates that SN was more sensitive than CBB to detect the differences among the genotypes in protein banding patterns. Grouping the cultivars based on their protein banding patterns stained with SN was not related to their susceptibility to PM. The results of this study indicate that cultivar specific bands may be useful as biochemical markers for cultivar identification or for seed purity tests.

Keywords: Flax genotypes, powdery mildew, SDS-PAGE.

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 (Mansour, 1998).

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 dependce 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 application. Furthermore, increasing cocern 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. Howevere, 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 the identification of flax genotypes with PM resistance.

Amino acid sequence of polypeptides (components of proteins) are dependent on nucleotide sequences of their coding genes; therefore, an analysis of protein variation among flax genotypes by electrophoresis, approximates an analysis of their genetic variation (Markert and Faulhaber, 1965).

Some attempts were made to differentiate among flax genotypes by using protein electrophoresis. For example, 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 identfied 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 of lodging and fungal diseases. Lapina (1989) reported that these patterns contained 15-22 bands, with the fewest being found in the petterns 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 differering in yield, resistance to lodging, and resistance to fungal diseases. They found that there were differences between protein banding patterns of the studied characters, and that patterns had bands in common and cultivar specific bands. There were 45 bands common to all the cultivars and 2 to 6 associated with the genotype of the particular seeds. They also reported that the cultivar, which had the widest range of economiclly useful traits had the highest number of bands in its pattern (71 bands).

The present investigation was initiated to determine whether flax genotypes, in particulary those with PM resistance, can be distinguished by their electrophoretic protein patterns separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique and stained with Coomassie Brillian Blue R-250 or with silver nitrate.

MATERIALS AND METHODS

Evaluation of flax genotypes for PM resistance

Experiments were conducted over four successive growing seasons, beginning in the fall of 1997. Experiments consisted of a randomized complete block design of 5 replicates (blocks). Plots were 2 x 3 m (6 m^2) and consisted of 10 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 incidence and disease severity were rated visually in the last week of April. Disease incidence was measured as the percentage of infected plants in a random sample of 100 plants/plot. 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 flax proteins:

Flax seeds were slightly ground and defatted by diethyl either or chloroform. After drying at room temperature, 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, mixed thoroughly with glass beads, and ground in liquid nitrogen to a fine powder. The powder was centrifugated at 19,000 rpm for 30 minurtes at 0°C. The protein content in the supernatant was estimated according to Bradford (1976) by using bovine serum albumin as a standard protein. If protein concentration was low, protein would be precipitated from the clarified supernatant by adding ammonium sulfate at 70% of saturation (60 g/100 ml) then kept in the refrigerator for 30 hrs. Pellets, collected by centrifugation at 11,000 rpm for 30 minutes, were resuspended in phosphate buffer pH 8.3 and subjected to dialysis for 24 hrs against the buffer and centrifugation at 11,000 for 30 minutes.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

Each supernatant was mixed with an equal volume of a solution consisting of (by volume) 64% buffer (0.15M Tris-HCl, pH 6.8); 20% glycerol; 6% sodium dodecyl sulfate (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 proteins) were subjected to electrophoresis in 15% polyacrylamide prepared in 0.1% SDS. Electrophoresis was conducted at room temperature (approximately 20 to 25°C), for 9 hrs on an 15% polyacrylamide gel with a 6% stacking gel, at 20 and 10 mA, respectively, until the dye reached the bottom of the separating gel. Electrophoresis was performed in a vertical slab mold (16.5 x 14.5 x 0.1 cm). Gels were stained with Coomassie Brilliant Blue R-250 (Laemmli, 1970 and Latorre *et al.*, 1995) or with silver nitrate (Sammons *et al.*, 1981).

Statistical analysis of the data

The experimental design of the field trials was a randomized complete block with five replications. 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. Correlation between disease incidence and severity was calculated by a computerized program.

Electrophoretic protein patterns obtained by SDS-PAGE from flax genotypes were culstered by the unweighted pair-group method based on arithmetic mean (UPGMA). Cluster analysis (Joseph *et al.*, 1992) was performed by a gel documentation system (Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631).

RESULTS and DISCUSSION

Environmental conditions in the four years of the present study were favorable for epiphytotic spread of the disease. This was apparent as these environmental conditions resulted in high levels of disease incidence (DI) and disease severity (DS) on all the tested genotypes. These high levels of DI and DS indicate that the genotypes were screened, for PM resistance, under high disease pressure. This high disease pressure is considered a prerequisite condition for any meaningful field evaluation for disease resistance. Line 3 showed the highest level of DI (81.9%), while line 366/2/1/2 showed the lowest level (62.6%). The other genotypes showed intermediate levels of DI between these two extremes (Table 1). As to DS, cultivar Sakha 1 showed the highest DS (86.8%), while line 366/2/1/2 showed the lowest DS (63.5%). The other genotypes were intermediate in DS. Although DI and DS were highly correlated in most of the years (Table 2), DI was more appropriate to evaluate susceptibility of the genotypes to PM because it was more precise (Rouse *et al.*, 1981) and more heritable than DS-that is, it was less affected by environmental conditions (Aly *et al.*, 2001).

Table 1: Susceptibility of ten flax genotypes to powdery mildew under field conditions in El-Gemmeiza.

Genotypes	Disease Incidence (%)[a]	Disease severity (%)[b]
Cultivar Giza 7	66.4[c] AB	67.0 BC
Line 1	79.4 AB	81.2 ABC
Line 3	81.9 A	85.0 AB
Line 366/2/1/2	62.6 B	63.5 C
Line 422/10/1/2	74.2 AB	80.9 ABC
Cultivar Giza 8	76.2 AB	75.8 ABC
Cultivar Sakha 1	79.9 AB	86.8 A
Cultivar Sakha 2	75.4 AB	77.9 ABC
Line 5	71.2 AB	77.6 ABC
Line 402/20/18/3	79.0 AB	87.3 AB

[a] Disease incidence was the percentage of infected plants in a random sample of 100 plants/plot.

[b] Disease severity was the percentage of infected leveas/plant in a random sample of 10 plants/plot.

[c] Means of 4 years. Percentage data were transformed into arch sine angles before carrying out the analysis of variance to produce approximately constant variance. Means followed by the same letter(s) were not significantly different ($P \leq 0.05$) according to Duncan's multiple range test..

Table 2: Correlation coefficients between severity and incidence of powdery mildew on ten flax genotypes evaluated under field conditions in El-Gemmeiza.

Year	Correlation coefficient (r)
1997/1998	0.589x[a]
1998/1999	0.850**
1999/2000	0.921**
2000/2001	0.853**

Linear correlation coefficient (r) was significant at P ≤ 0.10 (x) or P ≤ 0.01 (**).

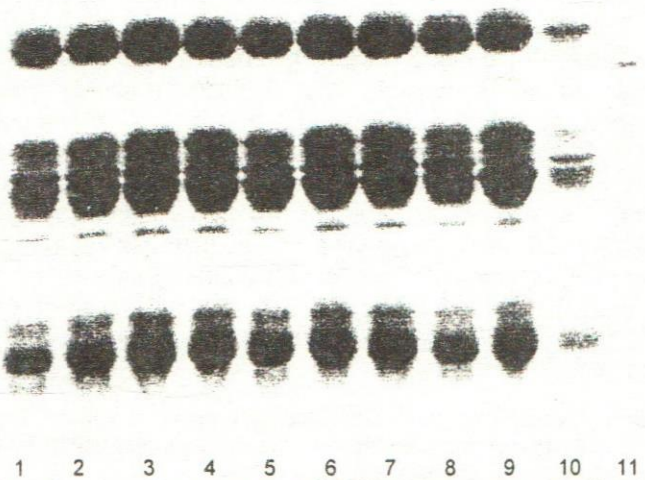
Electrophoretic banding patterns of Coomassie Brilliant Blue R-250 (CBB) are shown in Table 3 and Fig.1, while those stained with silver nitrate (SN) are shown in Table 4 and Fig. 3. Twenty five bands were detected by CBB. Of these bands, one (4.0%) was common to all the genotypes. The number of detected bands increased to thirty eight with SN. Of these bands, five (13.2%) were common to all the genotypes. All the genotypes showed variable increases in the number of bands when SN was used to visualize the protein patterns. These increases ranged from 33.3% (cultivar Sakha 2) to 150.0 (line 402/20/18/3 and cultivar Giza 7). The increases in the number of bands detected with SN indicate that SN was a highly sensitive visualization technique for detection of the small amounts of proteins, which cannot be seen with CBB. Generally, SN is 100-fold more sensitive than CBB (Anonymous, 2001).

Table 3: Protein banding patterns for flax genotypes obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250.

Band		Genotype[b]									
No.	MW[a]	1	2	3	4	5	6	7	8	9	10
1	72	-	-	-	-	-	-	-	-	-	+
2	71	-	-	-	-	-	+	+	+	+	-
3	70	-	-	-	+	-	-	-	-	-	-
4	69	-	+	-	-	-	-	-	-	-	-
5	63	-	+	-	-	-	-	-	-	-	-
6	58	-	-	-	-	-	-	-	+	+	-
7	57	-	-	-	-	+	-	-	-	-	-
8	54	-	-	-	-	-	-	+	+	+	+
9	53	-	-	-	-	+	+	-	-	-	-
10	52	+	+	+	+	-	-	-	-	-	-
11	43	-	-	-	-	-	-	-	-	-	+
12	34	-	-	-	-	-	-	+	-	+	+
13	33	+	+	+	+	+	+	-	+	-	-
14	30	-	-	-	-	-	-	-	+	-	-
15	29	-	-	+	-	+	-	+	-	+	-
16	28	-	+	-	-	-	-	-	-	-	-
17	24	-	-	-	-	-	+	+	+	+	+
18	23	+	+	-	-	+	-	-	-	-	-
19	21	+	-	-	-	-	-	-	-	-	-
20	20	+	+	+	+	+	+	+	+	+	+
21	18	-	-	-	-	+	+	+	+	+	+
22	17	+	+	+	+	-	-	-	-	-	-
23	13	-	-	-	-	-	-	-	-	+	+
24	12	-	-	-	-	-	-	+	-	-	-
25	11	+	+	+	+	+	+	-	+	+	+

[a] Molecular weight in kilodalton. [b] The genotypes were line 3 (1), line 5 (2), line 402/20/18/3 (3), cultivar Giza 7 (4), line 422/10/1/3 (5), cultivar Giza 8 (6), line 366/2/1/2 (7), cultivar Sakha 1 (8), cultivar Sakha 2 (9), and line 1 (10).

+ The designated band is present. -The designated band is absent.



1 2 3 4 5 6 7 8 9 10 11

Fig. (1): Protein banding patterns for flax genotypes obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250. Lanes 1 through 11 were line 1, cultivar Sakha 2, cultivar Sakha 1, line 366/2/1/2, cultivar Giza 8, line 422/10/1/3, cultivar Giza 7, line 402/20/18/3, line 5, line 3, and a marker, respectively.

A problem with proteins as biochemical markers for typing or classification of plants is the vast number, which can be generated. Faced with so much data, only sophisticated analysis can hope to draw meaningful conclusions. The ready availability of computers has made numerical taxonomy more accessible and some later studies on other organisms have proven useful (Manicom *et al.*, 1990). Therfore, in the present study we used a computerized gel documentation system for cluster analysis as an attempt to distinguish flax genotypes, in particular those with PM resistance, based on their protein profiles.

In the present study, protein bands were visualized by staining with CBB or with SN; however, it should be noted that the degree of staining is not necessarily directly proportional to the number of proteins in a given band. Staining intensity may be affected by the amino acid composition of the protein and its size as well as by the multiple proteins at a given site (Partridge *et al.*, 1984). Therefore, variation in intensity of bands was not used in cluster analysis, which was based entirely on presence or absence data.

Table 4. Protein banding patterns for flax genotypes obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver nitrate.

Band		Genotype [b]									
No.	MW[a]	1	2	3	4	5	6	7	8	9	10
1	97	-	+	-	-	-	-	-	-	-	-
2	96	-	-	-	-	+		+	-	-	-
3	86	-	-	-	-	+	+	+	-	-	-
4	83	+	-	-	-	-	-	-	-	-	-
5	82	-	+	+	-	-	-	-	-	-	-
6	81	-	-	-	+	+	+	+	+	+	+
7	79	+	-	-	-	-	-	-	-	-	-
8	78	-	-	-	+	-	-	-	-	-	-
9	77	-	-	-	-	-	+	+	-	-	-
10	71	+	-	-	-	-	-	-	-	-	-
11	70	-	+	+	+	+	+	+	+	+	+
12	65	+	-	-	-	-	-	-	-	-	-
13	64	-	+	-	-	-	-	-	-	-	-
14	63	-	-	+	+	+	+	+	+	+	+
15	59	+	-	-	-	-	-	-	-	-	-
16	58	-	-	+	-	+	+	+	+	+	+
17	57	+	-	-	-	-	-	-	-	-	-
18	56	-	+	+	+	+	+	+	+	+	+
19	52	+	-	-	-	-	-	-	-	-	-
20	51	-	+	+	-	+	+	+	+	+	+
21	50	-	-	-	+	-	-	-	-	-	-
22	46	+	+	+	+	+	+	+	+	+	+
23	41	+	+	+	+	+	+	+	+	+	+
24	35	-	+	-	-	-	-	-	-	-	-
25	32	+	-	-	-	-	-	-	-	-	-
26	31	-	-	-	-	-	-	-	-	+	-
27	30	-	+	+	-	+	+	+	+	+	+
28	29	-	-	-	+	-	-	-	-	-	-
29	27	-	+	-	-	-	-	-	-	-	-
30	25	-	-	-	-	-	-	-	+	+	+
31	24	-	-	-	+	+	+	+	-	-	-
32	23	+	+	+	-	-	-	-	-	-	-
33	21	+	+	+	+	+	+	-	+	+	+
34	19	+	-	-	-	+	+	+	-	-	-
35	18	-	+	+	+	-	-	-	-	-	-
36	17	+	+	+	+	+	+	+	+	+	+
37	16	+	+	+	+	+	+	+	+	+	+
38	15	+	+	+	+	+	+	+	+	+	+

[a] Molecular weight in kilodalton.

[b] The genotypes were line 3 (1), line 5 (2), line 402/20/18/3 (3), cultivar Giza 7 (4), line 422/10/1/3 (5), cultivar Giza 8 (6), line 366/2/1/2 (7), cultivar Sakha 1 (8), cultivar Sakha 2 (9), and line 1 (10).

\+ The designated band is present.

\- The designated band is absent.

Fig. 2 showed the phenogram constructed based on the similarity levels (SLs) generated from the cluster analysis of electrophoretic banding patterns of CBB shown in Table 3. The greater the SL, the more closely the genotypes were. The tested genotypes showed a very high overall SL (95.65), which indicates that CBB was insensitive technique to detect the differences in protein profiles among the genotypes.

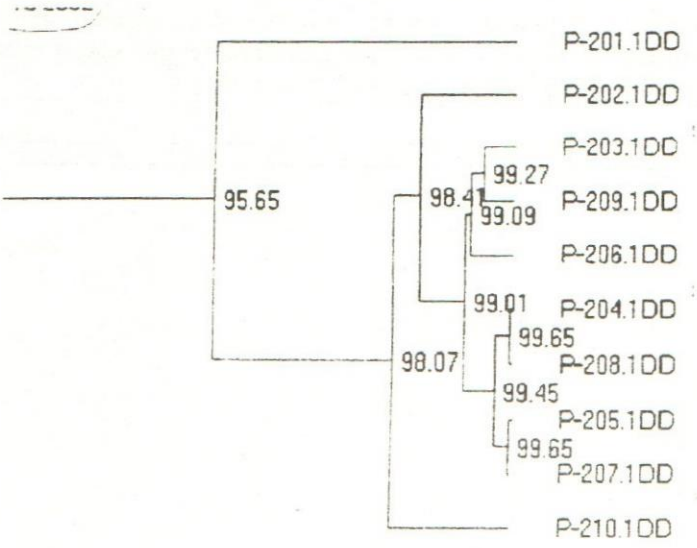


Fig. (2):Phenogram based on cluster analysis of electrophoretic banding patterns of flax genotypes obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250. The genotypes were line 3 (1), line 5 (2), lne 402/20/18/3 (3), cultivar Giza 7 (4), line 422/10/1/3 (5), cultivar Giza 8 (6), line 366/2/1/2 (7), cultivar Sakha 1 (8), cultivar Sakha 2 (9), and line 1 (10).

The phenogram of Fig. 4 was constructed based on the SLs generated from the cluster analysis of electrophoretic banding patterns of SN shown in Table 4. The overall SL among the genotypes was reduced to 66.56, which indicates that SN was more sensitive than CBB in detecting the differences in protein profiles among the tested genotypes. Line 3 constituted a separate subcluster remotely related to the other subcluster, which included all the other genotypes (SL = 88.18). Since these genotypes, except line 366/2/1/2, were not significantly different from line 3 in the DI, it was concluded that grouping the genotypes based on protein profiles did not match that based on susceptibility to PM. It is noteworthy that susceptibility of the genotypes to PM was judged based on only the DI for the previously mentioned considerations.

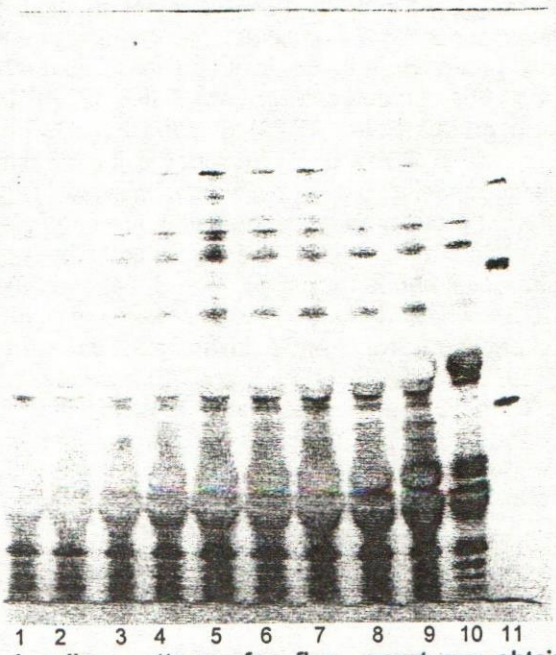


Fig. (3): Protein banding patterns for flax genotypes obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver nitrate. Lanes 1 through 11 were line 1, cultivar Sakha 2, cultivar Sakha 1, line 366/2/1/2, cultivar Giza 8, line 422/10/1/3, cultivar Giza 7, line 402/20/18/3, line 5, line 3, and a marker, respectively.

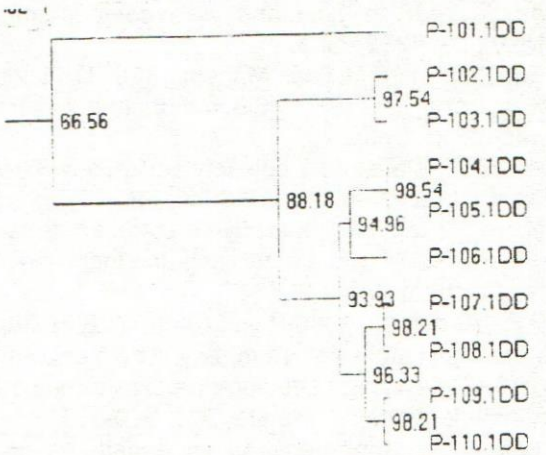


Fig. (4): Phenogram based on cluster analysis of electrophoretic banding patterns of flax genotypes obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver nitrate. The genotypes were line 3 (1), line 5 (2), line 402/20/18/3 (3), cultivar Giza 7 (4), line 422/10/1/3 (5), cultivar Giza 8 (6), line 366/2/1/2 (7), cultivar Sakha 1 (8), cultivar Sakha 2 (9), and line 1 (10).

Certain details in Table 4 and Fig. 4 are worthy of mention. The genotypes were placed in five distinct groups (subclusters). The first group included only line 3. The second group (SL = 97.54) included lines 5 and 402/20/18/3. The third group (SL = 94.96) included cultivar Giza 7, line 422/10/1/3, and cultivar Giza 8. The fourth group (SL = 98.21) included line 366/2/1/2 and cultivar Sakha 1. The fifth group (SL = 98.21) included cultivar Sakha 2 and line 1. There were cultivar specific bands by which cultivars, particularly those belonging to different groups, could be identified. For example, bands nos 4, 7, 10, 12, 15, 19, and 25 differentiated line 3 from any other genotype. There were only six differential bands (nos 1, 13, 14, 16, 24, and 29) between lines 5 and 402/20/18/3, which belonged to the same group; however, the differential bands between line 5 and cultivar Giza 7 increased to 13 (nos 1, 5, 6, 8, 13, 14, 20, 21, 24, 27, 29, 31, and 32) because they belonged to different groups. Thus, it seems reasonable to conclude that the observed cultivar specific bands may be useful as biochemical markers for cultivar identification or for seed purity tests.

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عدم وجود علاقة بين أنماط البروتين الناتجة من التفريد الكهربى للتراكيب الوراثية لنبات الكتان وقابلية هذه التراكيب للإصابة بمرض البياض الدقيقى

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أجريت دراسة مقارنة لأنواع البروتينات المستخلصة من عشرة تراكيب وراثية لنبات الكتان – تختلف فيما بينها من حيث درجة القابلية للإصابة بمرض البياض الدقيقى – وذلك باستعمال تقنية التفريد الكهربى للبروتين المفكك باستعمال مادة صوديوم دوديسيل سلفيت. استعملت مادة كوماسى بريليانت بلو أو مادة نترات الفضة لإظهار أنماط البروتين المتحصل عليها. استعمل أسلوب التحليل العنقودى لتصنيف هذه التراكيب الوراثية بناء على ما بينها من تماثل فى أنماط البروتين، وتم التعبير عن النتائج فى فينوجرام لكل طريقة من طريقتى الصبغ. أظهرت الدراسة أن درجة التماثل الكلى بين أنماط البروتين للتراكيب الوراثية كانت ٦٦,٥٦%. عندما استعملت نترات الفضة لصبغ البروتينات، فى حين ارتفعت درجة التماثل الكلى إلى ٩٥,٦٥% عندما استعملت كوماسى بريليانت بلو لصبغ البروتينات. تدل هذه النتيجة على أن نترات الفضة هى الأكثر حساسية للكشف عن الفروق فى انماط البروتين بين التراكيب الوراثية. أمكن باستعمال نترات الفضة تقسيم التراكيب الوراثية إلى مجموعات، إلا أن هذا التقسيم لم يكن مرتبطاً بدرجة قابلية التراكيب الوراثية للإصابة بمرض البياض الدقيقى. تدل نتائج الدراسة الحالية على أنه من الممكن استعمال حزم البروتينات، المميزة للتراكيب الوراثية، كمعلمات بيوكيميائية للتفرقة بين هذه التراكيب أو لإجراء اختبارات النقاوة للتقاوى.