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DIFFERENTIATION AMONG FLAX CULTIVARS BY RAPD ANALYSIS AND SUSCEPTIBILITY TO SOME PATHOGENIC FUNGI

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ABSTRACT

Random amplified polymorphic DNA (RAPD) analysis was used to evaluate the genetic diversity of 10 flax cultivars. Flax cultivars showed varying levels of susceptibility to a set of pathogenic fungi. The tested cultivars were analyzed with four random decamer primers using the polymerase chain reaction (PCR). All the primers detected varying levels of polymorphism in all the tested cultivars. Cluster analysis by the unweighted pair-group method of arithmetic means (UPGMA) placed cultivars in several groups based on their RAPD-PCR banding patterns with overall similarity levels ranged from 4.05 to 94.72%. UPGMA also placed studied cultivars in several groups based on the patterns of susceptibility to a set of pathogenic fungi. The present study demonstrated that flax cultivars could be identified by their RAPD-PCR banding patterns, combined with their patterns of susceptibility to a set of pathogenic fungi. These results could be practical value for cultivar identification or for seed purity tests.

Keywords: Flax genotypes, pathogenic fungi, polymerase chain reaction, RAPD analysis, genetic diversity.

INTRODUCTION

Flax (*Linum usitatissimum* L.) is the most important bast fiber crop in Egypt, it ranks second after cotton in terms of economic importance and production.Flax cultivars may be described as fiber, intermediate, or seed types on the basis of principle economic product. Cultivars developed for fiber production are relatively tall and produce low seed yields with lower than normal linseed oil content. Fiber producing cultivars are usually planted in dense stands to discourage branching. By contrast, flax cultivars grown primarily for oil production have been breeding high seed yield at the expense of decreased height, increased oil content of the seed, increased branching, and decreased fiber production and quality. Intermediate plant types attempt to maximize both fiber and seed yields (Dybing and Lay, 1981).

Seedling blight, root rot, and powdery mildew are among infectious diseases of flax in Egypt both on seed and fiber cultivars. They vary in importance with climate, cultural practice, cropping sequence, and cultivar, but each may assume economic significance in some regions and in some years (Dybing and Lay, 1981).

Rhizoctonia solani Kühn attacks flax at early stage of development, destroying the root and causing thinning or in severe infection, death of seedlings (Krylova, 1981). *R. solani* also causes root rot symptoms, which appear in plants after the flowering stage (Hartman, 1996).

There are various reports on the differences in susceptibility among flax genotypes to *R. solani*, but flax cultivars with resistance or immunity to *R. solani* are not yet known (Omran *et al.*, 1968; Anderson, 1977; Islam, 1992; and Bos and Parlevliet, 1995). Yellow-seeded varieties ate more prone to cracking, which renders them more susceptible to seedling blight and root rot than brown-seeded varieties (Hartman, 1996).

Fusarium spp. occur frequently among the fungal microflora associated with diseased flax roots and are a major cause of seedling blight and root rot in some flax-growing areas in Egypt (Aly *et al.*, 2011).

Powdery mildew 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. Currently, resistance to powdery mildew is not available in commercially grown flax cultivars in Egypt (Aly *et al.*, 2002 and Mansour *et al.*, 2003).

Genetic diversity among genotypes of plants can be evaluated with seed proteins and isozymes markets (Gepts, 1993). However, a large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner. This limits the use of isozymes, which may also lack adequate level of polymorphism (Tatineni *et al.*, 1996). Molecular genetic markers have been developed into powerful tools to analyze genetic relationships and genetic diversity.Restriction fragment length polymorphisms (RFLPs) can be used, but they are costly and time-consuming to evaluate. Random amplified polymorphic DNA (RAPD) is a useful technique to evaluate taxonomic identity and kinship (Hadrys *et al.*, 1992). RAPDs were shown to provide a level of resolution equivalent to RFLPs for determining genetic relationships among *Brassica oleracea* L. genotypes (Dos Santos *et al.*, 1994) and among *B. napus* L. breeding lines (Hallden *et al.*, 1994). The technical simplicity and speed of RAPD methodology is a principal advantage (Gepts, 1993). Ashry *et al.* (2002) used RAPD analysis to detect molecular markers for powdery mildew resistance in flax. RAPD analysis was also used by Aly *et al.* (2004) to quantify resistance of flax cultivars to powdery mildew.

The present investigation was initiated to determine whether flax cultivars can be distinguished by RAPD analysis in conjunction with their susceptibility to a set of pathogenic fungi included *F. oxysporum, F. moniliforme, F. solani, R. solani, Macrophomina phaseolina, Pythium* sp., and *O. lini*. These fungi were chosen because *O. lini* causes powdery mildew while the remaining fungi are involved in seedling blight and root rot of flax (Mansour, 1998 and Aly *et al.*, 2011).

MATERIALS AND METHODS

Susceptibility of flax cultivars to pathogenic fungi

(a) Soilborne fungi involved in seedling blight and root rot

Substrate for growth of each selected fungus was prepared in 500-ml glass bottles, each bottle contained 50 g of sorghum grains and 40 ml of tap

water. Contents of bottles were autoclaved for 30 minutes. Fungal inoculum, taken from one-week-old culture on PDA, was aseptically introduced into the bottles and allowed to colonize sorghum for three weeks. The present test was carried out by using autoclaved clay loam soil. Batches of soil were infested separately with inoculum of each fungus at rates of 50 g, 1 g, 50 g, 5 g/kg of soil for *Fusarium* spp., *R. solani*, *M. phaseolina*, and *Pythium* sp., respectively. Infested soil was dispensed in 10-cm-diameter clay pots and these were planted with 20 seeds per pot of each cultivar. Pots were randomly distributed on greenhouse benches. The greenhouse was equipped with a heating system assuring that the minimum temperature in the greenhouse was maintained at 28°C; however, due to the lack of a cooling system, the maximum temperature could not be controlled fluctuating from 30 to 35°C depending on the prevailing temperature during the day (the test was conducted in January and February 2012). Dead seedlings (combined pre-emergence and post-emergence damping-off) were recorded 45 days after planting.

(b) *O. lini*

Seeds of flax cultivars were planted on 15 November 2011 in autoclaved soil dispensed in 25-cm-diameter clay pots (20 seeds/pot). The pots were distributed outdoors in a randomized complete block design of four replications. Powdery mildew was allowed to develop naturally. Disease severity was rated visually at 15 April 2012 as percentage of infested leaves/plant in a random sample of 10 plants/pot (Nutter *et al.*, 1991).

Statistical analysis of susceptibility data

The experimental design of all the susceptibility tests was a randomized complete block with four replicates. Analysis of variance (ANOVA) of the data was performed MSTAT-C Statistical Package. Least significant difference (LSD) was used to compare between cultivar means. Correlation and cluster analyses were performed with the software package (SPSS 6.0).

DNA isolation and RAPD analysis

DNA was isolated from 500 mg of healthy fresh leaves of each cultivar (Table 1) using Qiagen kit for DNA extraction. The extracted DNA was dissolved in 100 µl of elution buffer. The concentration and purity of the obtained DNA was determined by using "Gene Quanta" system, Pharmacia Biotech. The purity of the DNA for all samples were between 90-97%. Concentration was adjusted to 6 ng/µl for all samples using TE buffer (pH 8.0).

Polymerase chain reaction (PCR) mixture was prepared with PCR bead tables (manufactured by Amessham Pharmacia Biotech.), which contained all the necessary reagent except the DNA template and the 10-mer primer.

The kits of Amessham Pharmacia Biotech include also the following primers:

RAPD Analysis primer 1: d(CGTGCGGGAA)-3
RAPD Analysis primer 2: d(GTTTCGCTCC)-3
RAPD Analysis primer 3: d(AACGCGCAAC)-3
RAPD Analysis primer 4: d(CCCGTCAGCA)-3

Table 1. Flax cultivars used in the present study.

Cultivar	Origin	Type	Pedigree
Giza 7	Local cultivar	Dual	Giza 5 x New River (USA)
Giza 8	Local cultivar	Dual	Giza 6 x Santa Catalina 6
Sakha 1	Local cultivar	Dual	Bombay x 1.1485
Sakha 2	Local cultivar	Dual	1.2348 (Hungary x I. Hira (India)
Bleinka	Holland	Fiber	Unidentified
Escalina	Holland	Fiber	Unidentified
Ilona	Holland	Fiber	Unidentified
Bombay	India	Oil	Unidentified
Gawhar	India	Oil	Unidentified
Gentiana	Romania	Oil	Unidentified

Thirty ng from each DNA extracted sample and 5 µl of the 10-mer random primer (15 ng/ml0 were added to a PCR bead tablets. The total volume was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows using OCR unit II Biometra: Denaturing at 95°C for 5 min, 45 cycles each consisted of the following steps: Denaturing at 95°C for 1 min, annealing at 36ŜC for 1 min, extension at 72°C for 2 min. Final extension was carried out at 72°C for 5 min and hold at 4°C.

Fine µl 6x tracking buffer (manufactured by Qiagen was added to 25 µl of the amplification product.

Amplification product analysis

The amplified DNA (15 µl) for all samples was electrophoresed using the electrophoretic unit WIDE mini-sub-cell GT (Bio-Rad) on 1% agarose containing 0.5 µg/ml ethidium bromide at 75 constant voltage, and determined with UV transilluminator.

Gel analysis

Gem was scanned for molecular weight (bp) and amount (%) of bands by the gel documentation system AAB (Advanced American Biotechnology, Fullerton CA, 92631). The different molecular weights of bands were determined against DNA standard (G317 A. Promega Inc., USA) with molecular weights 1000, 750, 500, 300, 150 and 50 bp.

RESULTS AND DISCUSSION

Significant variability was observed among the tested flax cultivars in susceptibility to the pathogenic fungi (Table 2). Correlation among cultivars in susceptibility to the pathogenic fungi is shown in Table 3. Of the 45 positive correlation coefficients (r_s) shown in the table, 22 (48.89%) were highly significant ($P < 0.01$).

Table 2. Susceptibility of flax cultivars to pathogenic fungi.

Cultivar	Susceptibility[a] to (%)						
	FO	FM	FS	RS	MP	P	OL
Giza 7	50.43[b]	62.22	32.27	53.50	32.42	42.26	92.45
Giza 8	52.58	63.79	33.30	54.67	33.52	43.68	93.27
Sakha 1	53.29	64.22	34.62	55.82	35.62	45.72	92.85
Sakha 2	50.46	61.53	32.75	52.48	36.75	46.82	93.65
Bleinka	70.26	66.34	39.64	72.16	81.24	90.42	83.26
Escalina	40.33	54.27	37.65	50.73	45.62	53.20	78.32
Ilona	42.87	55.81	38.21	51.81	46.81	54.62	79.45
Bombay	43.82	52.77	38.17	44.27	45.42	53.86	78.27
Gawhar	60.48	72.45	45.22	80.36	51.42	75.24	72.87
Gentiana	61.76	74.56	46.53	81.47	52.33	76.35	73.98
LSD ($P \leq 0.05$)	9.77	11.73	8.37	11.33	9.88	10.92	12.16
LSD ($P \leq 0.01$)	13.55	14.66	12.45	14.72	13.15	15.18	16.28

[a] Flax cultivars were tested for susceptibility to *F. oxysporum* (FO), *F. moniliforme* (FM), *F. solani* (FS), *R. solani* (RS), *M. phaseolina* (MP), *Pythium* sp. (P), and *O. lini* (OL).
[b] Mean of four replicates.

Table 3. Correlation among flax cultivars in susceptibility to pathogenic fungi.

Flax cultivar	Flax cultivar								
	1	2	3	4	5	6	7	8	9
1 Giza 7									
2 Giza 8	1.000**								
3 Sakha 1	1.000**	1.000**							
4 Sakha 2	0.994**	0.993**	0.994**						
5 Bleinka	0.329	0.329	0.344	0.403					
6 Escalina	0.893**	0.887	0.893**	0.928**	0.550				
7 Ilona	0.903**	0.897**	0.903**	0.938**	0.567	0.999**			
8 Bombay	0.905**	0.899**	0.904**	0.942**	0.567	0.993**	0.955**		
9 Gawhar	0.591	0.594	0.605	0.588	0.580	0.601	0.612	0.574	
10 Fentiana	0.594	0.597	0.608	0.589	0.568	0.598	0.609	0.570	1.000**

Pearson's correlation coefficient (r) is significant at P < 0.01 (**).

Fig. 1 showed the phenogram constructed based on the similarity levels (SL_s) generated by cluster analysis of RAPD banding patterns obtained by using primer no. 1. The greater the SL, the more closely the cultivars were related in their DNA banding patterns. This primer approximately placed the cultivars in three distinct groups based on their type at SL_s 98.65, 97.88, and 98.55%; however, this primer was unreliable to differentiate among cultivars because the overall SL generated by this primer was as high as 94.72%, that is, the primer detected a very low level of polymorphism among the cultivars.

Primer no. 2 (Fig. 2) produced the highest level of polymorphism among the cultivars. Hence, the overall SL was 4.05%. This primer was able to differentiate between some cultivars, which were placed in remotely related subcluster. For example, this primer showed a distant affinity between Giza 7 and Giza 8. Another example is the distant affinity between Giza 8 and Sakha 2. The primer also distinguished Bleinka from any other cultivar.

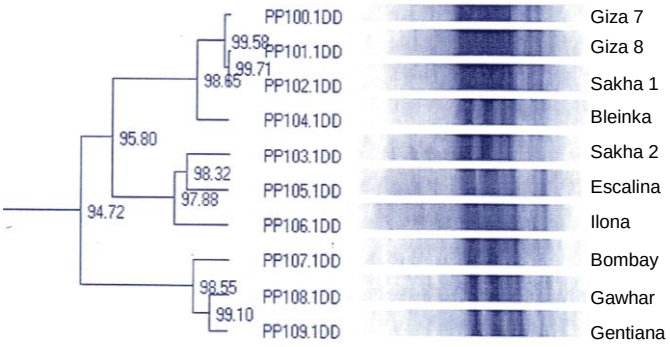

Fig. 1. RAPD banding patterns of flax cultivars obtained by the primer no. 1 and electrophoresed on agarose gel.

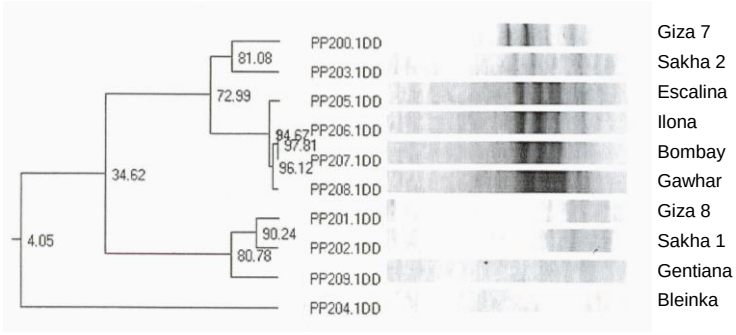

Fig. 2. RAPD banding patterns of flax cultivars obtained by the primer no. 2 and electrophoresed on agarose gel.

Primer no. 3 (Fig. 3) produced a reasonable level of polymorphism (the overall SL was 79.42%). Certain details in Fig. 3 are worthy of mention. This primer placed the cultivars in three distinct groups based on their type at SLs 96.51, 98.37, and 95.19%; however, Bleinka and Bombay were notable exceptions because Bleinka constituted a separate subcluster remotely related to the other cultivars and the patterns of Bombay were very close to those of Escalina and Ilona. Primer no. 3 was able to approximately differentiate among cultivars based on their type; however, it was unable to differentiate among cultivars within types due to the low level of polymorphism. Primer 3 easily distinguished Bleinka from any other cultivar.

Primer no. 4 (Fig. 4) was able to distinguish Ilona from each of Giza 7 and Sakha 1. Giza 8 was easily distinguished from any other cultivar.

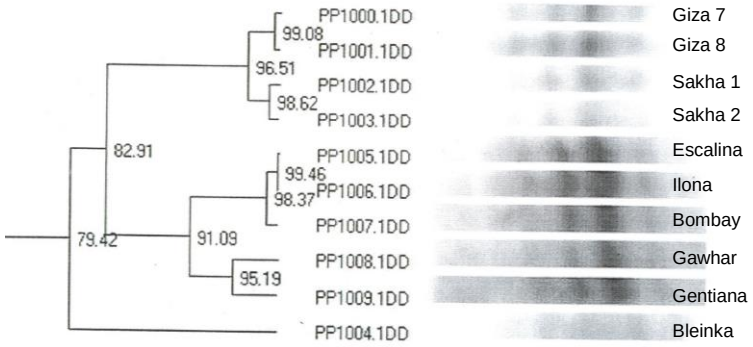

Fig. 3. RAPD banding patterns of flax cultivars obtained by the primer no. 3 and electrophoresed on agarose gel.

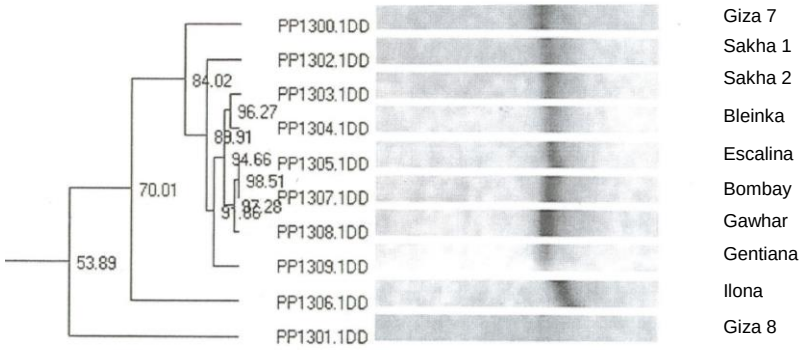

Fig. 4. RAPD banding patterns of flax cultivars obtained by the primer no. 4 and electrophoresed on agarose gel.

Another measure of genetic diversity is the taxonomic distance based on susceptibility of cultivars to some pathogenic fungi. Fig. 5 showed the phenogram constructed based on the dissimilarity levels (DL_s) generated from cluster analysis of cultivar susceptibility to some pathogenic fungi (Table 1). The smaller the DL (distance), the more closely the cultivars were related in their susceptibility patterns. In this phenogram, susceptibility pattern placed the cultivars in three distinct groups related to their type; however Bombay and Bleinka were notable exceptions because Bombay was placed with fiber cultivars and Bleinka was placed in a separate subcluster unrelated to the remaining cultivars. The phenogram in Fig. 5 indicates that susceptibility pattern was able to differentiate between dual cultivars and oil cultivars except Bombay. On the other hand, susceptibility pattern was unreliable to differentiate between dual cultivars and fiber cultivars except Bleinka. It is worthy of mention that grouping the cultivars based on their susceptibility patterns was identical to their grouping based on RAPD patterns obtained by the primer no. 3.

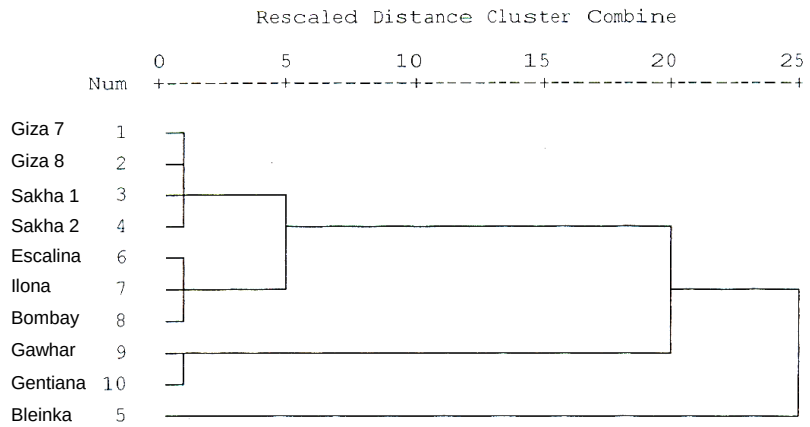

Fig. 5. Phenogram based on average linkage cluster analysis of susceptibility of 10 flax cultivars to 7 pathogenic fungi.

The present study demonstrates that flax cultivars could be identified by their RAPD-PCR banding patterns, combined with their patterns of susceptibility to a set of pathogenic fungi. These results could be of practical value for cultivar identification or for seed purity tests.

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التفرقة بين أصناف الكتان بإستعمال تقنية التضاعف العشوائى لمناطق متباينة من الحمض النووى DNA والقابلية للإصابة ببعض الفطريات الممرضة

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إستعملت تقنية التضاعف العشوائى لمناطق متباينة من الحمض النووى DNA لتقييم درجة التنوع الوراثى فى ١٠ أصناف من الكتان ، تتباين الاصناف فيما بينها من حيث درجة القابلية للإصابة بمجموعة من الفطريات الممرضة جيدة. إستعملت أربعة بوادئ عشوائية لإجراء تفاعل البلمرة المتسلسل على الحمض النووى المستخلص من الأصناف موضع الدراسة. وأشارت النتائج أن تمكنت جميع البوادئ من إحداث تضاعف للحمض النووى لجميع الأصناف. تم إستعمال التحليل العنقودى لتصنيف الأصناف إلى مجموعات بناءً على مابينها من تمائل فى أنماط الحمض النووى. أظهرت نتائج الدراسة أن درجة التماثل الكلى بين أنماط الحمض النووى للأصناف تراوحت مابين ٤,٠٥ إلى ٩٤,٧٢% حسب نوع البادئ المستخدم. أوضحت نتائج التحليل العنقودى أيضاً لتقسيم الأصناف إلى مجموعات بناءً على مابينها من تباين فى أنماط القابلية للأصابة بمجموعة من الفطريات الممرضة ، تدل نتائج الدراسة الحالية على أنه من الممكن الجمع مابين إستعمال أنماط الحمض النووى وإستعمال أنماط القابلية للأصابة وذلك لتعريف أصناف الكتان أو لإجراء إختبارات النقاوة للتقاوى.

قام بتحكيم البحث

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