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Differentiation between Resistance and Susceptibility of Fiax Cultivars to Powdery Mildew by Molecular Techniques

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ABSTRACT

Six flax cultivars (*Linum usitatissimum* L.) were evaluated for powdery mildew resistance in outdoor experience. Cultivars wilden, Bombay and Dakota were resistant (disease severity was 62.80, 58.77 and 69.70% respectively), while cultivars Cortland, sofie, and C.I. 2008 were susceptible (disease severity was 98.10, 97.90, and 87.07% respectively). DNA was extracted from cultivar leaves and subjected to random amplified polymorphic DNA (RAPD) analysis by using five random primers. Primer no.5 was partially successful in differentiating between susceptible cultivars sofie and C.I.2008 and the other resistant cultivars. Extracted DNA was subjected to inter – simple sequence repeat (ISSR) by using five random primers. Primer no.9 was successful in differentiating between the resistant cultivars and the susceptible ones. Primers no. 7 and no. 10 were partially successful in differentiation between some susceptible and resistant cultivars.

INTRODUCTION

Flax (*Linum usitatissimum* L.) is one of the oldest crops, being domesticated over 7000 years ago possibly in Mediterranean or Indian region (Maiti *et al*., 2011). Flax is infected by a number of diseases, of which powdery mildew (PM) is next in importance to rust. The disease is caused by the obligate parasite *Odium lini* Skoric. The fungus attacks all the aboveground parts of flax Aly *et al*., 1994). The disease (PM) is easily recognized by the white powdery growth of fungus on infected portions of the flax plant. Symptoms often first appear on the upper leaf surface but can also develop on lower leaf surfaces. Heavily infected leaves dry up; wither and die. Early infections may cause complete defoliation of flax plant. PM develop well in environments with high humidity and moderate temperature (Mansour, 1998). The disease can be effectively managed by cultivation of resistant cultivars. Aly *et al*., (2004) reported that all commercially grown flax cultivars in Egypt were susceptible to the disease. 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 (Ashry *et al*., 2002). There is a need to improve PM resistance in flax cultivars through detection and introgression of PM resistance genes. The development of PM. resistant cultivars through conventional breeding is a long and costly process. Moreover, suitable conditions for disease incidence are required for field evaluation, limiting the screening to only once per year. The greenhouse screening for PM resistance is also subjected to seasonal availability of inoculum because of the obligate nature of the pathogen (Ashry *et al*., 2002). Therefore, using molecular markers linked to the PM resistance gene for the indirect selection is an efficient alternative. It allows rapid selection at all growing season, there by significantly shortening the breeding process (Poolsawat *et al*., 2017). Various molecular markers have been used in several crop breeding programs (Arunakumari *et al*., 2016, Kassa *et al*., 2017, Poolsawat *et al*., 2017, and Zhang *et al*., 2017).

Molecular markers such as random amplified polymorphic DNA (RAPD) and inter – simple sequence repeat (ISSR) have an excellent potentiality to assist selection in breeding programs because they identify desirable genotypes independent from environmental variation. Consequently, marker – assisted selection can offer an efficient and rapid mean to identify PM – resistance gene (s) (Ashry *et al.,* 2002).

The objective of the present study was to evaluate some flax cultivars to PM infection and to study the possibility of utilizing RAPD and ISSR techniques in differentiation between resistant and susceptible some flax cultivars.

MATERIALS AND METHODS

Evaluation of flax cultivars for powdery mildew resistance:

Six flax cultivars were evaluated for powdery mildew resistance in pots (30 cm diameter) in outdoor experiment in 22 December 2015. The tested cultivars were Wilden, Bomby, Dakota, Cortland, Sofie, and C.I. 2008. Three replicates (pots) were used for each tested cultivar (50 seed/pot). After four weeks of planting the seedlings reduced to fifteen plant/pot. The reducing seedlings of each cultivar were used for RAPD and ISSR analysis. Disease severity was measured as percentage of infected leaves/plant in 25 April 2016 (Aly *et al*., 1994).

DNA Extraction:

Young and freshly leaves of each flax cultivar used to extract DNA of each cultivar. DNA extraction was performed as described by Dellaporta *et al*., (1983). The resulted pellets containing DNA were re-suspended in 8 Mμ TE (10mM Tris –HCL pH 8 and 1 mM EDTA) buffer. Qualities and quantities of DNA samples were determined and electrophorased.

RAPD Technique:

In order to obtain clear reproducible amplification products, different preliminary experiments were carried out in which a number of factors were optimized. These factors included PCR temperature cycle profile and concentration of each of template DNA, primer, MgCl2 and Taq polymerase. Five random DNA oligonucleotides primers were independently used to generate reproducible polymorphic DNA products according to Williams *et al*., (1990). Table (1) lists the base sequences of these DNA primers that produced informative polymorphic bands. The PCR amplification was performed in a 25 µl reaction volume containing the following: 2.5 µl of dNTPs (2.5 mM), 1.5 µl of MgCl2 (25 mM), 2.5 µl of 10x buffer, 2.0 µl of primer (2.5 µm), 2.0 µl of template DNA (50ng/µl), 0.3 µl of Taq

polymerase (5U/µl) and 14.7 µl of sterile dd H_2o. The reaction mixtures were overlaid with a drop of light mineral oil per sample. Amplification was carried out in Techni Tc-S12 PCR System. The reaction was subjected to one cycle at 95°c for 5 minutes followed by 35 cycles at 94°c for 30 seconds, 37°c (annealing temperature) for 30 seconds, and 72°c for 30 seconds, then a final cycle of 72°c for 12 minutes. PCR products were run at 100 V for one hour on 1.4% agaors gels to detect polymorphism between different cultivars under study. After electrophoresis, the RAPD patterns were visualized with UV transilluminator. RAPD markers were scored from the gels as DNA fragments present (1) or absent (0) in all lanes. PCR amplification was performed using five random 10 mer arbitrary primers synthesized by Operon biotechnologies, Inc. Germany.

Table 1. List of the primers' names used in random amplified polymorphic DNA (RAPD)

Primer No.	Name	Sequence
No. 1	OP-A01	5´CAA TCG CCG T 3`
No. 2	OP-A07	5´GAA AGG GGT G 3`
No. 3	OP-A10	5´CAA TCG CCG T 3`
No. 4	OP-B09	5´GTA GAC CCG T 3`
No. 5	OP-Z03	5´TCG GAT CCG T 3`

ISSR – PCR Technique:

Five random DNA oligonucleotide primers were in dependently used according to Williams *et al.,* (1990) in PCR reaction as previously mentioned in RAPD technique except annealing temperature was 57°c for 30 seconds instead of 37°c for 30 seconds in RAPD technique.

PCR amplification was performed using five ISSR primers synthesized by Operon biotechnologies, Inc. Germany (Table 2).

Table 2. List of the primers' names used in inter – simple sequence repeat (ISSR)

Primer No.	Name	Sequence
No. 6	14-A	5´CTC TCT CTC TCT CTC TTG 3`
No. 7	44-B	5´CTC TCT CTC TCT CTC TGC 3`
No. 8	HB-08	5´GAG AGA GAG AGA GG 3`
No. 9	HB-13	5´GAG GAG GAG GC 3`
No. 10	HB-15	5´GTG GTG GTG GC 3`

Statistical Analysis:

DNA bands generated by each primer were counted and their molecular sizes were compared with those of DNA marker (100bp DNA Ladder composed of eleven individual DNA fragments). The bands scored from DNA profiles generated by each primer were pooled together. The presence (+) or absence (-) of each DNA band was treated as binary character in a data matrix to calculate genetic similarity and to construct dendrogram tree among flax cultivars calculation was achieved using Dice similarity coefficients (Dice, 1945) as implemented in computer program SPSS.10.

RESULTS AND DISCUSSION

Pathogenicity test:

Flax cultivars could be divided in two distinct groups. The first group included cultivars wilden, Bomby, and Dakota which were resistant to PM. The infection in these cultivars ranged from 58.77% to 69.70%. The second group included the susceptible cultivars Cortland, Sofie, and C.I 2008 (Fig. 1 and Table 3). The most susceptible cultivar was Cortland with infection 98.10%, while the most resistant cultivar was Bomby (58.77%). Mansour (1998) reported that PM occurs annually in all flax production areas in Egypt. All commercially grown flax cultivars are susceptible to the disease, although field observations indicated that some experimental lines were more susceptible than others (Mansour 1998).

Fig. 1. Powdery mildew symptoms on the resistant cultivar (Dakota) and the susceptible cultivar (Sofie)

Table 3. Flax cultivars used their geographic Origen, and their reaction class to powdery mildew.

No.	Cultivar	Disease Severity %	Reaction Class	Geographic Origen
1	Wilden	62.80[a]	Resistant	USA
2	Bomby	58.77	Resistant	USA
3	Dakota	69.70	Resistant	USA
4	Cortland	98.10	Susceptible	USA
5	Sofie	97.90	Susceptible	Belgium
6	C.I 2008	87.07	Susceptible	USA

LSD for cultivar (p≤ 0.05) = 11.23

[a] Mean of the three replicates (pots).

Random Amplified Polymorphism DNA (RAPD):
Primer No. 1 = OP-A01

The tested cultivars were placed in two subclusters. The first one (Distance = 0.0) included the resistant cultivars Wilden, Bomby and Dakota. However, the susceptible cultivar Cortland was included in the same subcluster. The second subcluster (Distance = 6.5) included the two susceptible cultivars Sofie and C.I. 2008. Evidently, this primer was not a reliable one to differentiate between resistance and susceptibility because it placed cultivar Cortland (susceptible) with the resistant cultivars. (Figs. 2 and 3 and Table 4)

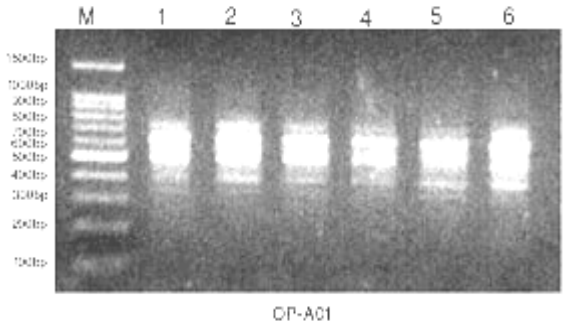

Fig. 2. RAPD banding patterns of flax cultivars obtained by the primer No. 1 (OP-A01) and electrophoresed on agarose gel.

Table 4. Number and distribution of RAPD banding patterns of flax cultivars obtained by the primer No. 1 (OP-A01) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	741	+	+	+	+	-	-
2	678	-	-	-	-	+	+
3	613	+	+	+	+	+	+
4	605	-	-	-	-	-	+
5	491	+	+	+	+	-	-
6	441	-	-	-	-	+	+
7	367	+	+	+	+	+	+

+ = DNA fragments present - = DNA fragments absent

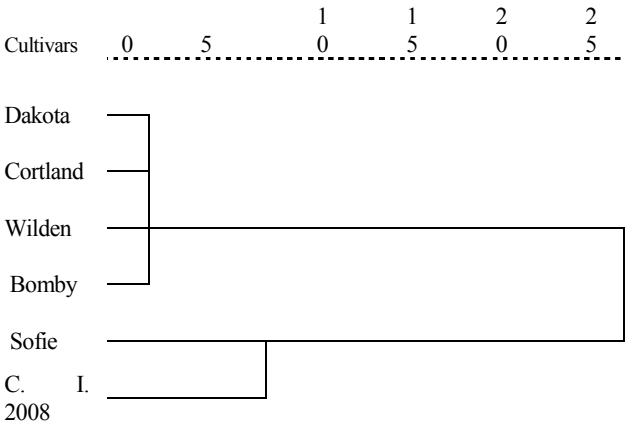

Fig. 3. Phenogram based on average linkage cluster analysis of RAPD banding patterns of flax cultivars obtained by the primer No. 1 (OP-A01) and electrophoresed on agarose gel.

Primer No. 2 = OP–A07

This primer placed the tested cultivars in three sub clusters. The first one (Distance = 7.5) included cultivars Bomby, Sofie, and C.I. 2008. Within this subcluster the resistant cultivar Bomby and the susceptible cultivar Sofie showed identical DNA profile. The second subcluster (Distance = 17.5) included the resistant Dakota and the susceptible Cortland. Cultivar Wilden made up a separate subcluster unrelated to the other cultivars in DNA profile. This primer can be used to differentiate cultivar Wilden in seed purity tests. (Figs. 4 and 5 and Table 5)

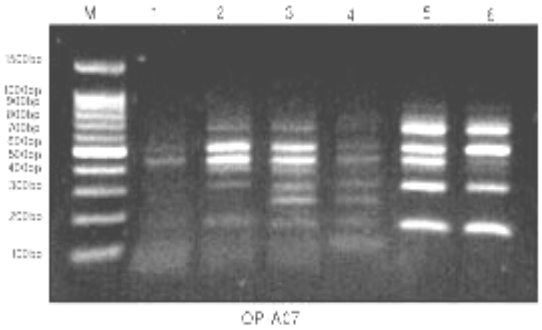

Fig. 4. RAPD banding patterns of flax cultivars obtained by the primer No. 2 (OP-A07) and electrophorесed on agarose gel.

Table 5. Number and distribution of RAPD banding patterns of flax cultivars obtained by the primer No. 2 (OP-A07) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	684	-	+	+	+	+	+
2	511	+	+	+	+	+	+
3	445	+	+	+	+	+	-
4	371	-	-	+	-	-	-
5	309	-	+	+	+	+	+
6	249	-	-	+	+	-	-
7	183	-	+	+	+	+	+
8	131	-	-	-	+	-	-

+ = DNA fragments present - = DNA fragments absent

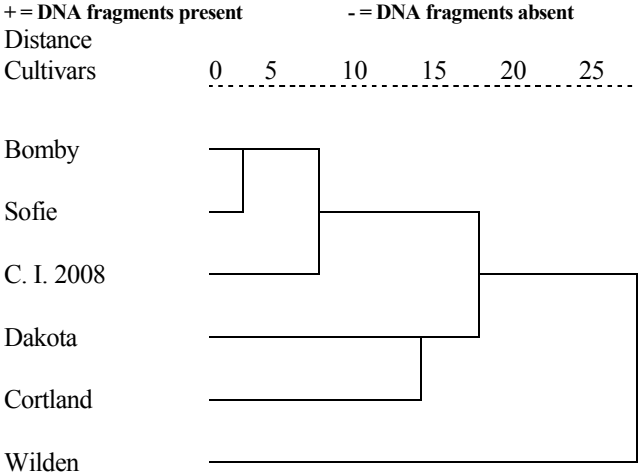

Fig. 5. Phenogram based on average linkage cluster analysis of RAPD banding patterns of flax cultivars obtained by the primer No. 3 (OP-A10) and electrophoresed on agarose gel.

Primer No. 3 = OP–A10

This primer was not able to differentiate between Dakota (resistant) and Sofie (susceptible) because they showed identical DNA profile. This primer also placed the two resistant Wilden and Dakota in two unrelated subclusters. The same conclusion was true for the two susceptible C.I. 2008 and Sofie (Figs 6 and 7 and Table 6).

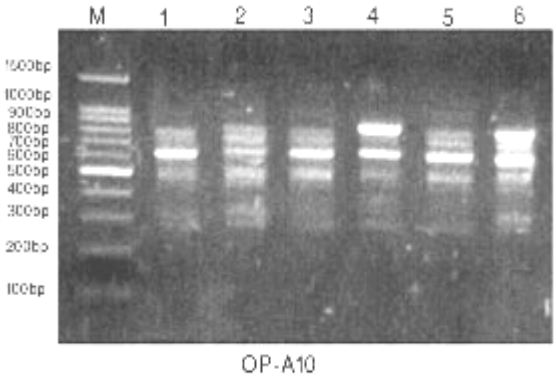

Fig. 6. RAPD banding patterns of flax cultivars obtained by the primer No. 3 (OP-A10) and electrophoresed on agarose gel.

Table 6. Number and distribution of RAPD banding patterns of flax cultivars obtained by the primer No. 3 (OP-A10) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	838	-	+	+	+	+	-
2	784	+	-	-	-	-	+
3	734	-	+	+	-	+	+
4	606	+	+	+	+	+	+
5	485	+	+	+	+	+	+
6	312	-	+	-	-	-	+
7	266	+	+	+	+	+	+

+ = DNA fragments present - = DNA fragments absent

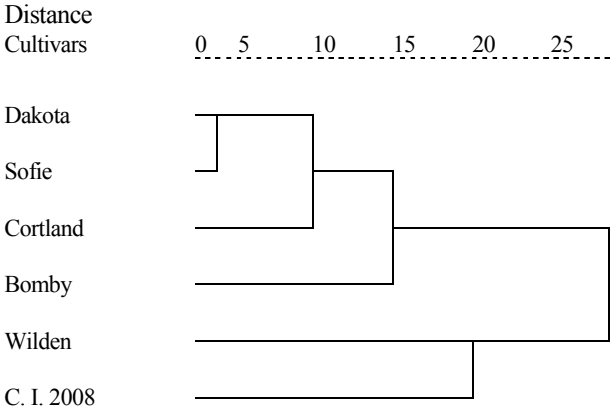

Fig. 7. Phenogram based on average linkage cluster analysis of RAPD banding patterns of flax cultivars obtained by the primer No. 3 (OP-A10) and electrophoresed on agarose gel.

Primer No. 4 = OP-B09

This primer placed flax cultivars in two unrelated subclusters. Each subcluster included both resistant and susceptible cultivars (Figs 8 and 9 and Table 7).

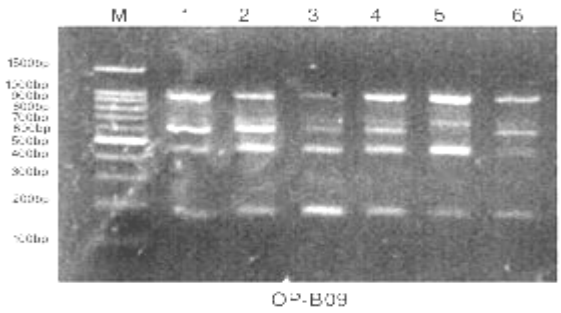

Fig. 8. RAPD banding patterns of flax cultivars obtained by the primer No. 4 (OP-B09) and electrophoresed on agarose gel.

Table 7. Number and distribution of RAPD banding patterns of flax cultivars obtained by the primer No. 4 (OP-B09) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	976	+	+	+	+	+	+
2	653	-	+	-	+	+	-
3	569	+	+	+	+	-	+
4	438	+	+	+	+	+	+
5	155	+	+	+	+	+	+

+ = DNA fragments present - = DNA fragments absent

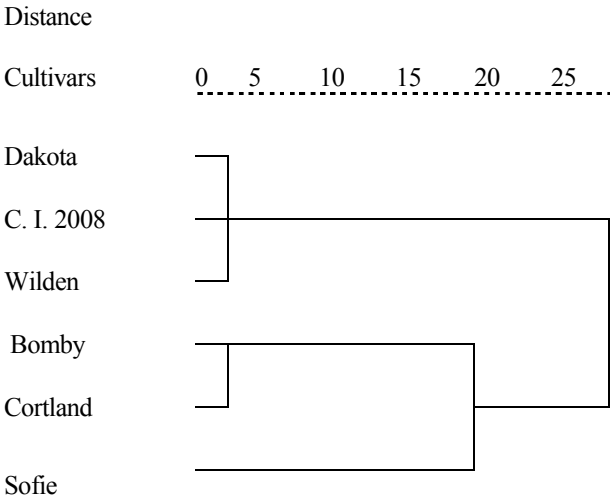

Fig. 9. Phenogram based on average linkage cluster analysis of RAPD banding patterns of flax cultivars obtained by the primer No. 4 (OP-B09) and electrophoresed on agarose gel.

Primer No. 5 = OP–Z03

This primer was partially successful in differentiating between the susceptible cultivars Sofie and C.I. 2008 and the other resistant Wilden, Bomby, and Dakota which were placed in unrelated subclusters. However, this primer unable to differentiate between the susceptible cultivar Cortland and the resistant cultivars Bomby and Wilden (Figs 10 and 11 and Table 8).

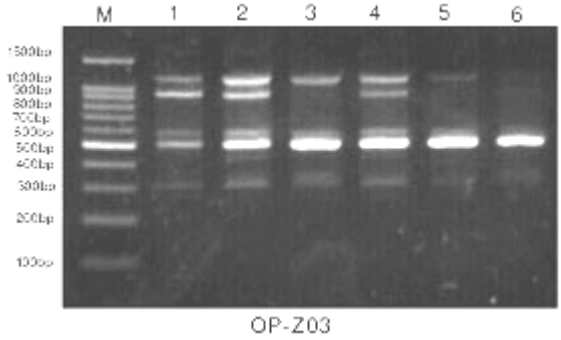

Fig. 10. RAPD banding patterns of flax cultivars obtained by the primer No. 5 (OP-Z03) and electrophoresed on agarose gel.

Table 8. Number and distribution of RAPD banding patterns of flax cultivars obtained by the primer No. 5 (OP-Z03) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	1180	+	+	+	+	-	-
2	984	+	+	-	+	-	-
3	593	+	+	+	+	-	-
4	531	+	+	+	+	+	+
5	304	+	+	+	+	-	-

+ = DNA fragments present - = DNA fragments absent

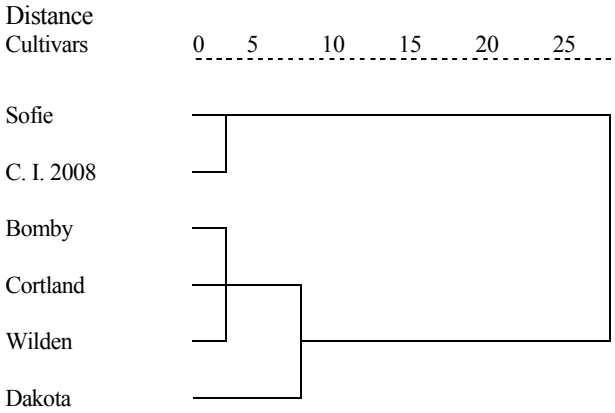

Fig. 11. Phenogram based on average linkage cluster analysis of RAPD banding patterns of flax cultivars obtained by the primer No. 5 (OP-Z03) and electrophoresed on agarose gel.

Inter–Simple Sequence Repeat (ISSR)

Primer No. 6 = 14-A

This primer placed all the tested cultivars in one group. That is this primer was unable to detect any differences among the cultivars in DNA profiles (Figs 12 and 13 and Table 9).

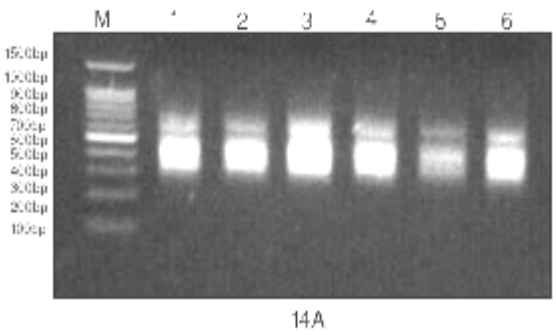

Fig. 12. ISSR banding patterns of flax cultivars obtained by the primer No. 6 (14A) and electrophoresed on agarose gel.

Table 9. Number and distribution of ISSR banding patterns of flax cultivars obtained by the primer No. 6 (14A) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	575	+	+	+	+	+	+
2	374	+	+	+	+	+	+
3	262	+	+	+	+	+	+

+ = DNA fragments present - = DNA fragments absent

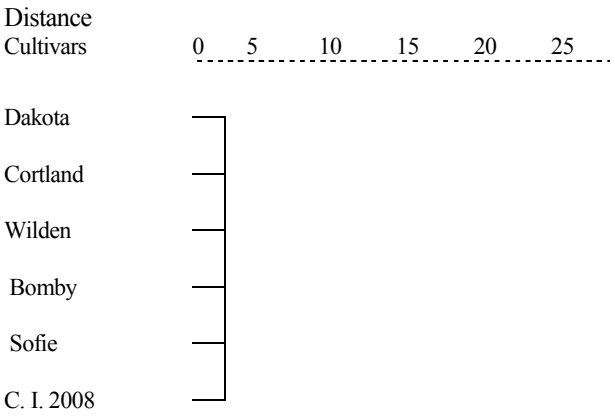

Fig. 13. Phenogram based on average linkage cluster analysis of ISSR banding patterns of flax cultivars obtained by the primer No. 6 (A14) and electrophoresed on agarose gel.

Primer No. 7 = 44-B

This primer was partially successful in differentiating between the susceptible cultivars (Cortland and Sofie) and other resistant cultivars. This primer was less reliable to differentiate between the resistant cultivars (Bomby, Dakota and Wilden) and the susceptible cultivar C.I. 2008 because it was included with them in the same subcluster (Distance = 20). (Figs 14 and 15 and Table 10).

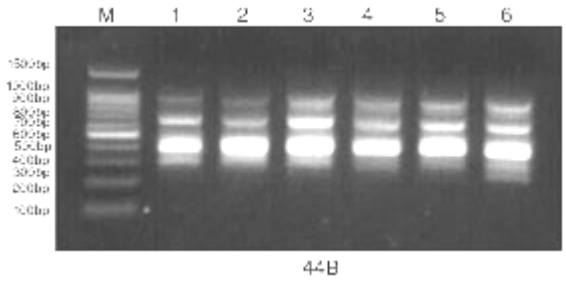

Fig. 14. ISSR banding patterns of flax cultivars obtained by the primer No. 7 (44B) and electrophoresed on agarose gel.

Table 10. Number and distribution of ISSR banding patterns of flax cultivars obtained by the primer No. 7 (44B) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	941	+	+	+	+	+	+
2	631	+	+	+	+	+	+
3	384	+	+	+	+	+	+
4	292	+	+	+	-	-	+
5	213	-	-	-	-	-	+

+ = DNA fragments present - = DNA fragments absent

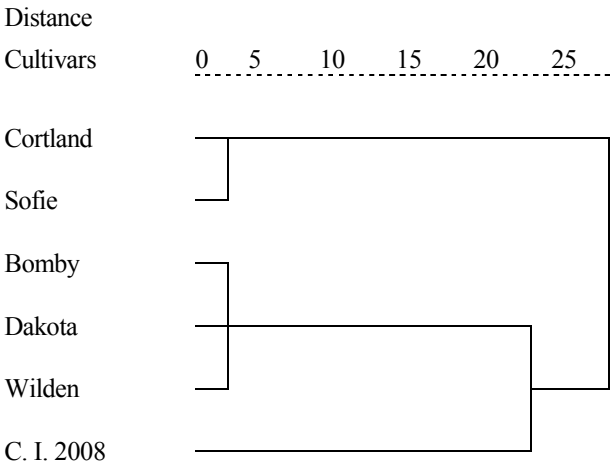

Fig. 15. Phenogram based on average linkage cluster analysis of ISSR banding patterns of flax cultivars obtained by the primer No. 7 (44B) and electrophoresed on agarose gel.

Primer No. 8 = HB–08

This primer placed all the flax cultivars in one group so it was unable to differentiate among cultivars in DNA profiles. (Figs 16 and 17 and Table 11).

Primer No. 9 = HB–13

This primer divided flax tested cultivars to two distinct unrelated groups. One group included the susceptible cultivars (Cortland, Sofie, and C.I. 2008) and the other group included the resistant cultivars Wilden, Bomby, and Dakota, which indicated that this primer can be used to differentiate between resistant and susceptible flax cultivars to powdery mildew (Figs. 18 and 19 and Table 12).

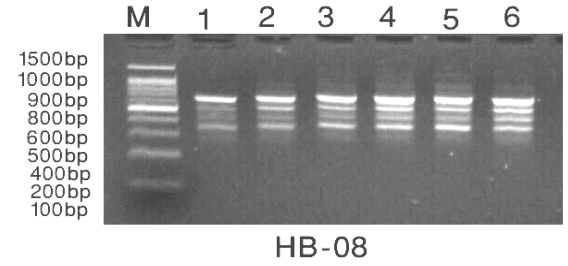

Fig. 16. ISSR banding patterns of flax cultivars obtained by the primer No. 8 (HB-08) and electrophoresed on agarose gel.

Table 11. Number and distribution of ISSR banding patterns of flax cultivars obtained by the primer No. 8 (HB-08) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	689	+	+	+	+	+	+
2	556	+	+	+	+	+	+
3	456	+	+	+	+	+	+
4	379	+	+	+	+	+	+

+ = DNA fragments present - = DNA fragments absent

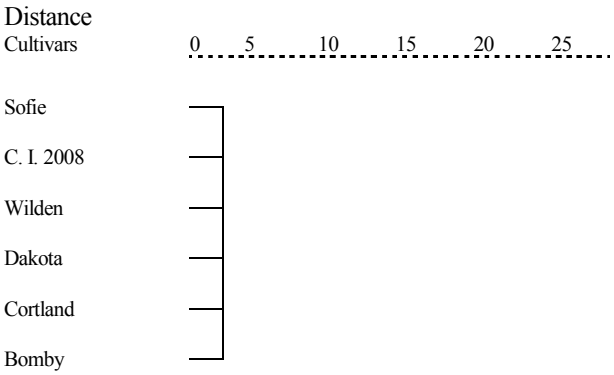

Fig. 17. Phenogram based on average linkage cluster analysis of ISSR banding patterns of flax cultivars obtained by the primer No. 8 (HB-08) and electrophoresed on agarose gel.

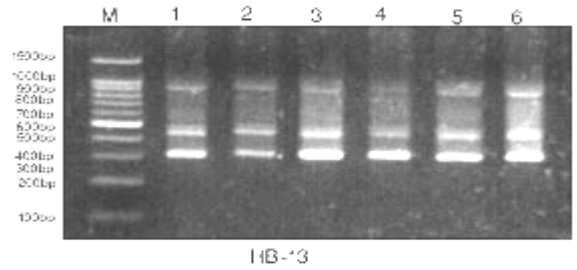

Fig. 18. ISSR banding patterns of flax cultivars obtained by the primer No. 9 (HB13) and electrophoresed on agarose gel.

Table 12. Number and distribution of ISSR banding patterns of flax cultivars obtained by the primer No. 9 (HB13) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	942	+	+	+	-	-	-
2	829	-	-	-	+	+	+
3	492	+	+	+	+	+	-
4	443	+	+	+	+	+	+
5	302	+	+	+	+	+	+

+ = DNA fragments present - = DNA fragments absent

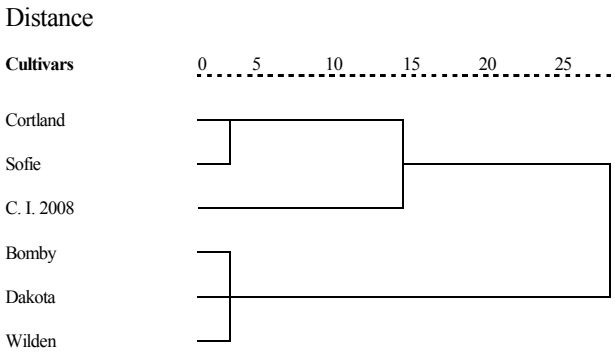

Fig. 19. Phenogram based on average linkage cluster analysis of ISSR banding patterns of flax cultivars obtained by the primer No. 9 (HB13) and electrophoresed on agarose gel.

Primer No. 10 = HB–15

This primer was partially successful in differentiation between the two susceptible cultivars (Sofie and C.I. 2008) and all the resistant cultivars Wilden, Bomby, and Dakota, which were placed in another unrelated subcluster (Figs. 20 and 21 and Table 13).

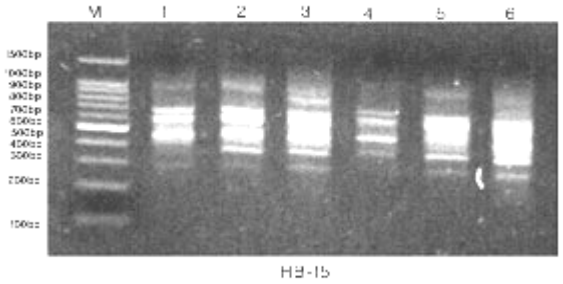

Fig. 20. ISSR banding patterns of flax cultivars obtained by the primer No. 10 (HB15) and electrophoresed on agarose gel.

Table 13. Number and distribution of ISSR banding patterns of flax cultivars obtained by the primer No. 10 (HB15) and electrophoresed on agarose gel.

Band No.	M.W. (bp)	Wilden	Bomby	Dakota	Cortland	Sofie	C. I. 2008
1	946	+	+	+	-	-	-
2	838	-	-	-	-	+	+
3	750	-	-	+	-	-	-
4	657	+	+	-	+	-	+
5	600	-	-	+	-	-	-
6	568	+	+	-	-	+	-
7	531	-	-	+	+	-	+
8	475	-	-	-	-	+	+
9	425	+	+	-	-	-	-
10	417	-	-	+	+	+	-
11	352	-	-	-	-	-	+
12	333	-	+	+	+	-	-
13	295	-	-	-	-	+	+
14	252	+	+	+	-	-	-
15	228	-	-	-	-	+	+

+ = DNA fragments present - = DNA fragments absent

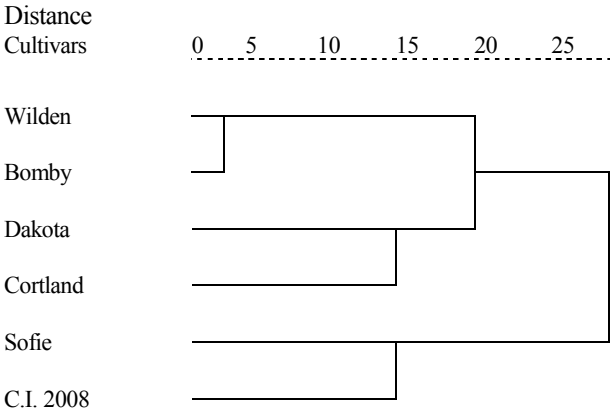

Fig. 21. Phenogram based on average linkage cluster analysis of ISSR banding patterns of flax cultivars obtained by the primer No. 10 (HB15) and electrophoresed on agarose gel.

In general, our results are in agreements with some previous studies, which indicated the usefulness of molecular markers in differentiation between resistant and susceptible genotypes. For example, Ashry *et al*., (2002), reported that RAPD analysis can be used as molecular marker for PM resistance in flax by using one of six random primers. Hussein *et al*., (2010) reported that RAPD analysis used in combination with pathogenicity test could be used in screening flax genotypes for PM resistance. Fu (2005), Uysal *et al*., (2010), Rajwade *et al.,* (2010) Singh *et al.,* (2014) and Satya and Chakrabort (2015) used RAPD and ISSR analysis in studying the genetic diversity of flax. Poolswat *et al*., (2017) found that ISSR marker is highly efficient tool for mapping PM resistance gene in mungbean.

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التفرقة بين المقاومة والقابلية للاصابة بمرض البياض الدقيقى فى أصناف الكتان باستعمال التقنيات الجزيئية

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أُختبرت ستة أصناف من الكتان لمقاومة الإصابة بمرض البياض الدقيقى فى تجربة أصص خارج الصوبة تحت ظروف العدوى الطبيعية. الأصناف ويلدين وبومباى وداكوتا كانت مقاومة للإصابة حيث كانت نسبة اصابتها ٦٢.٨٠ % و٥٨.٧٧ ٪ و٦٩.٧٠ ٪ على التوالى وفى حين كانت الأصناف كورتلاند وصوفى وسى أى ٢٠٠٨ قابلة للإصابة بنسبة ٩٨.١٠ ٪ و ٩٧.٩٠ ٪ و ٨٧.٠٧٪ على التوالى. إستُخلص الحامض النووى دى أن أيه من أوراق الأصناف واستخدامه فى عمل التضاعف العشوائى لمناطق متباينة من الحامض النووى بإستخدام خمسة بوادىء عشوائية . نجح البادىء رقم ٥ جزئياً فى التفريق بين الصنفين القابلين للأصابة صوفى وسى أى ٢٠٠٨ والأصناف الآخرى المقاومة (ويلدين وبومباى وداكوتا). كما تم استخدام الحامض النووى فى إجراء تحليل التتابع الداخلى المتكرر البسيط بإستخدام خمسة بوادىء نجح البادىء رقم ٩ فى التمييز بين الأصناف المقاومة والأصناف القابلة للأصابة . نجح كل من البادىء رقم ٧ ورقم ١٠ فى التمييز جزئياً بين بعض الأصناف القابلة للأصابة والأصناف المقاومة .