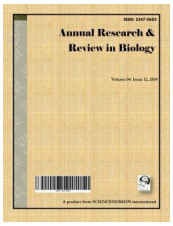

Annual Research & Review in Biology

21(2): 1-12, 2017; Article no.ARRB.38495
ISSN: 2347-565X, NLM ID: 101632869

Assessment of Flax Varieties for Drought Tolerance

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HAM, SAM and MAMEE participated in all experiments. Author SAM designed the study and wrote the first draft of the manuscript. Author MESO performed the statistical analysis and contributed in the writing of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2017/38495
Editor(s):
(1) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Martín Maria Silva Rossi, Argentina.
(2) Albino Maggio, University of Napoli Federico II, Italy.
(3) Mueen Alam, The Islamia University of Bahawalpur, Pakistan.
Complete Peer review History: http://www.sciencedomain.org/review-history/22358

Original Research Article

Received 29th November 2017
Accepted 14th December 2017
Published 19th December 2017

ABSTRACT

Introduction: Flax (*Linum usitatissimum* L.) is one of the ancient yields cultivated for dual purposes (oil and fibers). Drought stress plays an important role in the productivity of the flax crop in the world.
Methodology: A laboratory experiment on early seedling growth stage was conducted to evaluate the drought tolerance of Asile and Gentiana flax varieties. Three concentrations of polyethylene glycol (PEG10,000) 5, 10 and 15% were used as drought levels compared with the control (0 level). After ten days of cultivation, germination indices were recorded.
Results: The germination percentage was decreased with the increasing of PEG10,000 in both varieties. Highly significant variance was shown in percentage of germination and shoot length between Gentiana and Asile flax varieties. SDS-PAGE banding patterns indicated that PEG10,000 induced the drought tolerant in the varieties which led to the appearance or disappearance of some polypeptides due to water deficit and depending on the flax variety. Isoenzyme activities of polyphenol oxidase (PPO) and peroxidase (POX) did not differ between control and drought stress conditions in Asile, but differed in Gentiana variety. However, the RAPD-PCR assay exhibited

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polymorphism between the stressed plants and control. A total number of 75 fragments were amplified by seven decamer primers ranged from 90 to 1400 bp. Forty-four alleles were monomorphic bands (58.67%), while thirty-one loci were polymorphic (41.33%).
Conclusion: The results showed that Asile (the Indian) variety was more tolerant to drought than Gentiana (the Romanian) variety which had the highest percentage of germination.

Keywords: Linum usitatissimum L.; polyethylene glycol; SDS-PAGE; isozyme; RAPD-PCR.

1. INTRODUCTION

Drought is one of the prevailing environmental conditions that induce adverse effects on the plant growth. The role of drought stress is more for limitation the growth and productivity of the crop than other stresses, especially that the recent climate changes increase the risk of this situation [1,2,3,4].

Flax (*Linum usitatissimum* L.) is one of the ancient harvests cultured for the dual objectives of fiber and oil [5]. Other economic products such as animal feed-stock and omega 3 fatty acids for human diet supplements are also obtained from flax. In flax growing regions, the yield is vulnerable to drought and high temperature where the effects are pronounced at seed germination. The primary growth of the seedling is one of the most sensible stages which is a reaction to the environmental stress. This stage has a determining role on suitable and the final function of the plant [6]. Development of stress tolerant varieties/cultivars has been a major objective of many breeding programs. However, success has been limited by inadequate screening techniques and lack of genotypes that illustrate apparent differences in response to well-defined environmental stresses. Yield has been the foremost criteria for such programs and is a very complex trait in terms of genes number controlling it. This trait is also largely influenced by the environmental factors that cause selection for such less effective traits. Another trouble is those ascribable major environmental factors, in significant decrease of heritability of crop and its components. Drought related traits can be evaluated in the off-season or in controlled laboratory conditions in early generations, which could be a cost effective and still potential approach [7].

The objective of this research was to evaluate the response of two flax varieties to drought stress by treatment with polyethylene glycol 10,000 at the germination and seedling stages under laboratory conditions.

2. MATERIALS AND METHODS

2.1 Plant Materials

The seeds of two oil flax varieties: (Asile) an Indian variety and (Gentiana) a Romanian variety were imported from India and Romania, respectively to be the plant material of this investigation. The seeds were surface sterilized with sodium hypochlorite (1%) solution for one minute, then washed three times with distilled water. Each five sterilized seeds were put on a filter paper moistened with one of three drought levels, according to supplementing the Hoagland solution with polyethylene glycol (PEG): 5, 10 and 15% of PEG 10000 as well as the control (only Hoagland's solution). Each filter paper was put in a tissue culture tube and incubated at 25±1°C in the light for ten days for germination (germination was considered when the radical reached 2 mm long). A completely randomized design with eight replications was used. Five seedling parameters: shoot and root lengths, shoot/root ratio (S/R) [length of the shoot to the length of the root] and the shoot and root weights were recorded in the last day with five samples for each replicate [8,9]. The germination percentage was assessed according to the following formula:

Germination % = Number of the germinated seeds/ Total number of the seeds x 100.

2.2 Electrophoretic Analysis of Protein by SDS-PAGE

SDS-PAGE was done according to the method described by Laemmli [10] and modified by Studier [11].

2.3 Polyphenol Oxidase (PPO) and Peroxidase (POX) Isoforms

Antioxidant enzymes peroxidase (POX) and polyphenol oxidase (PPO) were extracted of bulked samples of each treatment for both varieties according to Stagemann et al. [12].

PPO and POX isozymes were separated by Native-polyacrylamide gel electrophoresis (Native-PAGE). The activities of POX and PPO were determined according to Brown; Baaziz et al. [13,14].

2.4 Extraction of Genomic DNA

Bulk samples from young plant leaves of both flax varieties were soaked in liquid nitrogen for DNA extraction using 2% (CTAB) Cetyltrimethyl ammonium bromide [15].

2.5 RAPD Analysis

A total of seven primers were used to amplify DNA [16] (manufactured by Bioneer, New technology certification from ATS Korea). The total reaction mixture was 25 µl contained 10X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs mixed, 10 *p*mol primer, 1.25 U *Taq* polymerase and about 150 ng genomic DNA. RAPD-PCR amplification was performed in the thermal cycler (Biometra Inc., Germany). The temperature profile was as follows: an initial denaturation at 94°C for 3 min; followed by 35 cycles of denaturation temperature 94°C for 5 min; annealing temperature 37°C for 1 min and extension temperature 72°C for 1 min, final extension at 72ºC for 5 min.

Amplification products were separated on a 1.5% agarose gel containing 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) and 0.5 µg/ml ethidium bromide at 90 V. Gels were analyzed by UVI Geltec version 12.4, 1999-2005 (USA).

2.6 Statistical Analysis

The data were analyzed by ANOVA procedure of program SPSS (1995) statistical procedures version 21 (Chicago, USA) (www.spss.com).

3. RESULTS AND DISCUSSION

3.1 Varieties Performance

Mean values of germination percentage and five seedling growth parameters affected by three drought levels. In addition, the control plants of Gentiana and Asile flax varieties were shown in Fig. 1 and Table 1. Germination and all the seedling parameters decreased with increasing drought levels except shoot/root rate increased with increasing drought levels in Gentiana variety but clearly disturbed in Asile variety. However, analysis of variance showed highly significant variance between varieties for the percentage of germination and shoot length (Table 2). Percentage of germination and all parameters (except shoot weight) affected significantly by the drought levels. Meanwhile, shoot length and shoot/root ratio showed highly significant interaction between flax varieties and drought levels.

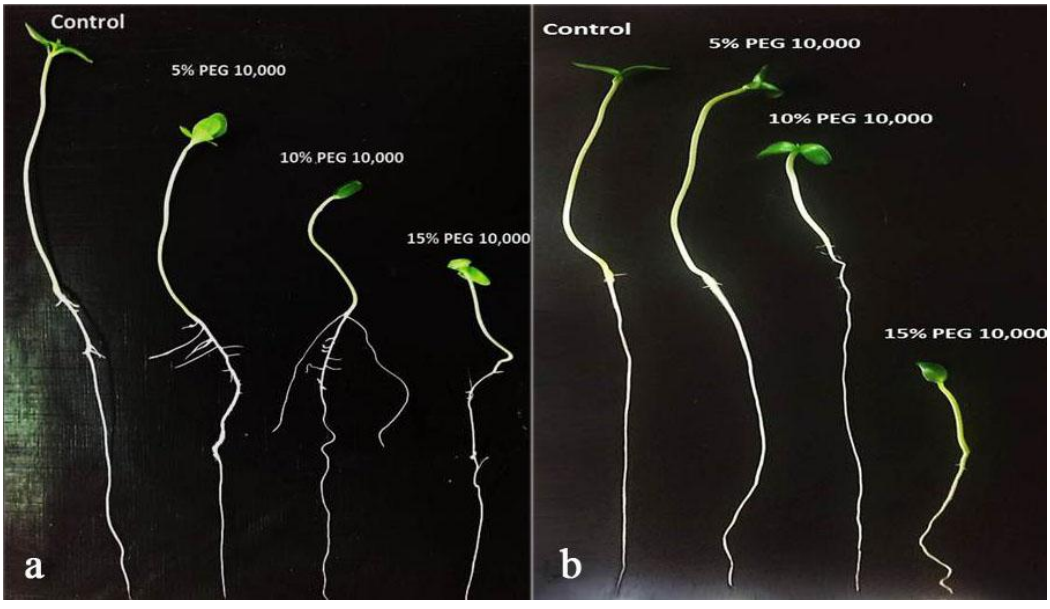

Fig. 1. Effect of osmotic stress induced by different concentrations of PEG10,000 on two flax varieties (a) Asile and (b) Gentiana, compared with the control

Different performance was found between varieties and their interaction with drought levels reflects the different genetic background between Gentiana and Asile flax varieties. Many authors found different performance owing to flax cultivars or varieties [17,18,19,20,21,22,23, 24,25,26].

3.2 Protein Electrophoresis by SDS-PAGE

SDS-PAGE was analyzed to characterize protein patterns involved in water stress response in the two flax varieties (Fig. 2 and Table 3). A total number of 22 polypeptides were showed heterogeneity among the drought stressed and the control plants in both flax varieties with molecular weights (MWs) ranged from 15 to 250 kDa, whereas ten subunits were polymorphic with 45.45% polymorphism. Twelve bands were monomorphic with 54.55% were recorded. The highest number of bands (20 bands) was recorded in Gentiana seeds treated with 15% PEG10,000, followed by 19 bands in Asile seeds stressed with 5% PEG10,000 and Gentiana seeds treated with 10% PEG10,000. However, the lowest number of polypeptides (13 polypeptides) was detected in Asile seeds stressed at 10% PEG10,000. Asile and Gentiana stressed with 15 and 5% PEG10,000, respectively were equally in the bands number (16 polypeptides). On the other hand, only one unique marker with MW +28 kDa was found in the control of Gentiana variety. In contrast, negative marker of -15 kDa disappeared in 10% PEG10,000 of Asile variety and also in the others. Moreover, one subunit of +40 kDa appeared only with 5% PEG10,000 in both varieties (Fig. 2 and Table 3).

SDS-PAGE banding patterns showed that water stress induces some bands to the appearance or disappearance response to water deficit and depending on the flax variety. Therefore, detection of proteins by SDS-PAGE revealed tolerance for drought and showed significant differences between flax varieties. Drought induced some proteins directly or indirectly in cellular adaptations to stress. These proteins can help in comprehension of the molecular detection of the alterations in gene expression of flax varieties under water deficit stress. In summary, drought stress induced variations in the synthesis of protein. Hence, proteins accumulation was found in the drought-stressed plants of two flax varieties, which could protect the plants from dehydration damage. The results were in conformity with the study of Dubey and Rani; Dai et al. Zala et al. [27,28,29] who found that SDS-PAGE analysis showed that water stress induced the production of two new proteins based on the wheat cultivar. Many of investigators mentioned that decrease protein concentration in barley plants stressed with PEG due to the protein synthesis rate is a low. In addition, enzymes involved in the amino acids and the protein syntheses are denaturated. Riccardi et al. [30] found that water stress stimulated expression of protein not linked to this stress, but related to reactions against the cell damage.

3.3 Enzyme Activity

The values of PPO and POX activities were constitutive different in the control and drought stress of both flax varieties (Fig. 3). Asile variety scored four PPO and three POX isoforms with different *Rf* values ranged from (0.283 to 0.900) and (0.395 to 0.887), respectively. There were no differences in the activities of PPO and POX isozymes between the control and all stressed seeds of Asile variety (Fig. 3).

Meanwhile, Gentiana variety generated four PPO and three POX isoenzymes banding with *Rf* ranging of (0.283 to 0.900) and (0.395 to 0.887), respectively. One isoform of PPO with *Rf* value 0.424 was absent in the control, but appeared in all induced stress, showing its function in the drought tolerant. In contrast, one isoform of *Rf* 0.900 was recorded in the control and disappeared in the others. Furthermore, one isoform of *Rf* 0.859 was absent only in 15% PEG10,000. Related to POX detection, two isoforms with *Rf* 0.395 and 0.505 appeared in the control and all stressed seeds, while one allele with *Rf* 0.538 appeared only in 5 and 15% PEG10,000. Addition to, the isoform of 0.887 *Rf* which singly appeared in the control seeds. In Gentiana variety, PPO and POX were more affected by drought stress than in Asile variety (Fig. 3).

The result showed that the activity of PPO and POX isoforms varied depending on the variety and drought level. These results can be applied as biochemical parameters for the screening of drought tolerant flax varieties in the arid areas. This result agrees well with Oskuei et al. [31] who mentioned that change in POX isozymes activity depending on the wheat genotypes under drought stress. Also, the response of isoenzymes to drought wasn't the same for all isoforms of the antioxidant enzymes in the wheat genotypes, as POX isoforms displayed the significant variations in the genotypes tolerance to drought. In addition, the changes in antioxidant enzyme

activities are related to tolerance of wheat genotypes to the abiotic stress. The antioxidant defense capacity and the variation of individual enzymatic activities during stress were depended on the plant cultivar/variety. Isozymes analysis is important because it can support to realize how the water stress can affect the different subcellular compartments [32].

3.4 RAPD-PCR Profiles

A total number of 75 fragments were amplified by seven dominant RAPD-PCR primers (10.7 loci per primer), ranging from 90 to 1400 bp (Fig. 4 and Table 4). Forty-four alleles were monomorphic bands (58.67%), while thirty-one loci were polymorphics (41.33%). The number of alleles per primer varied from three by RAPD-3 to 17 by RAPD-7. One the other hand, primer RAPD-7 recorded the maximum number of polymorphism (58.82%), followed by primer RAPD-1 (50%). Moreover, Primer RAPD-2 scored the lowest number of polymorphism (22.22%). Besides, 11 out of the 75 were unique bands (14.67%). Primer RAPD-7 exhibited the highest number of molecular markers (five), followed primer RAPD-6 (three). However, primers RAPD-1, RAPD-3, and RAPD-5 scored (one) specific band. Three markers were shown in the Gentiana control with molecular sizes -145, -788 and -990 bp, using primer RAPD-7. Two amplified fragments of +463 and +610 bp were scored in the concentration 5% PEG10,000 for Asile variety by primer RAPD-6.

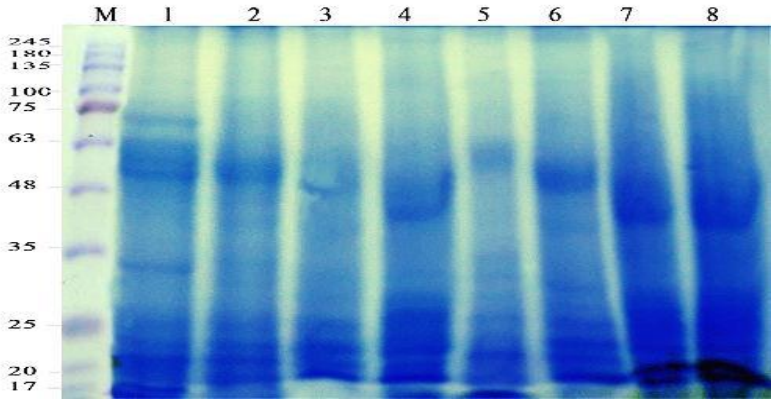

Fig. 2. SDS-PAGE banding in the portion of two flax varieties affected by different drought levels

Lane M= Protein marker, lanes 1= control of Asile variety, lanes 2, 3 and 4= represent 5, 10 and 15% PEG10,000, respectively) of Asile variety. Lane 5= control of Gentiana variety, lanes 6, 7 and 8 = represent 5, 10 and 15% PEG10,000, respectively) of Gentiana variety.

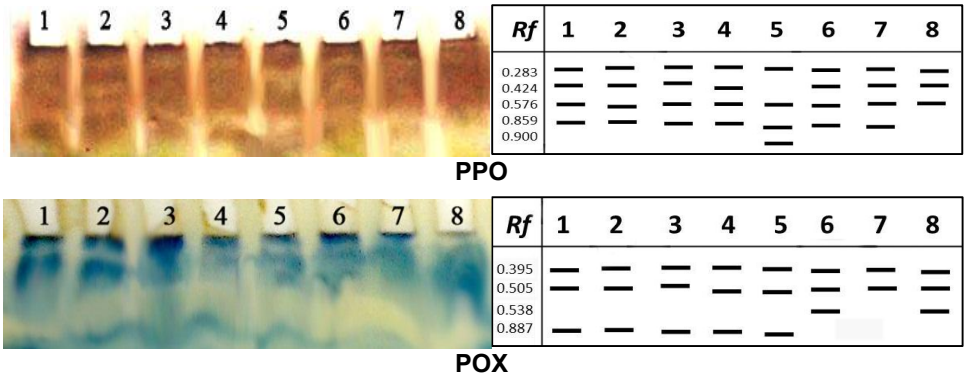

Fig. 3. Effect of drought stress on isozyme patterns of PPO and POX in two flax varieties, designations of isoforms are shown to the left of the zymograms

Lanes 1= control of Asile variety, lanes 2, 3 and 4= represent 5, 10 and 15% PEG10,000, respectively) of Asile variety. Lane 5= control of Gentiana variety, lanes 6, 7 and 8 = represent 5, 10 and 15% PEG10,000, respectively) of Gentiana variety.

Table 1. Mean values of germination and five seedling growth parameters affected by different drought levels in two flax varieties

Varieties	Drought levels	Germination (%)	Shoot length (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)	Shoot/ Root rate
Gentiana	Control	90.00±3.78	6.71±0.68	7.74±0.95	0.06±0.00	0.03±0.00	1.09±0.07
	5%	82.50±2.50	5.58±0.48	3.41±0.53	0.04±0.01	0.01±0.00	2.29±0.45
	10%	57.50±8.81	2.63±0.35	1.58±0.26	0.03±0.00	0.01±0.00	2.40±0.37
	15%	42.50±7.01	1.90±0.18	0.80±0.10	0.02±0.00	0.01±0.00	3.08±0.56
Asile	Control	100.00±0.00	10.51±0.52	7.05±0.67	0.09±0.01	0.03±0.00	1.65±0.10
	5%	100.00±0.00	7.36±0.75	2.74±0.54	0.04±0.01	0.01±0.00	3.21±0.52
	10%	95.00±3.27	4.05±0.52	2.68±0.38	0.03±0.00	0.01±0.00	1.77±0.19
	15%	72.50±11.30	1.59±0.27	0.81±0.10	0.02±0.00	0.01±0.00	2.18±0.36

Table 2. Analysis of variance (MS) of germination and five seedling growth parameters affected by different drought levels in two flax varieties

S.O.VA.	DF	Germination (%)	Shoot length (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)	Shoot/ Root rate
Varieties (V)	1	9025.00 **	44.57 **	0.05	0.002	0.0001	0.00
Drought levels (D)	3	4650.00 **	152.12 **	130.13 **	0.007	0.0007 **	6.37 **
V x D Interaction	3	608.33	11.43 **	2.84	0.001	0.0000	3.18 **
Residual	**56**	**285.71**	**2.01**	**2.16**	**0.016**	**0.0001**	**1.09**

*** Highly significant*

Table 3. SDS-PAGE analysis of two flax varieties treated with different drought levels

No.	MW (kDa)	Asile variety				Gentiana variety			
		Control	5% PEG	10% PEG	15% PEG	Control	5% PEG	10% PEG	15% PEG
1	250	1	1	1	1	1	1	1	1
2	248	0	1	0	1	0	0	1	1
3	136	1	1	1	1	1	1	1	1
4	75	1	1	0	0	0	1	1	1
5	63	1	0	0	0	1	0	0	1
6	60	1	1	1	1	0	0	1	1
7	55	1	1	1	1	1	1	1	1
8	48	1	1	1	1	1	1	1	1
9	45	0	1	0	1	0	1	1	1
10	40	0	1	0	0	0	1	0	0
11	35	0	0	0	0	1	0	1	1
12	33	1	1	0	0	1	0	1	1
13	30	1	1	1	1	1	1	1	1
14	28	0	0	0	0	1	0	0	0
15	27	1	1	1	1	1	1	1	1
16	25	1	1	1	1	1	1	1	1
17	24	1	1	1	1	1	1	1	1
18	23	1	1	1	1	1	1	1	1
19	20	1	1	1	1	1	1	1	1
20	19	1	1	1	1	1	1	1	1
21	17	1	1	1	1	1	1	1	1
22	15	1	1	0	1	1	1	1	1
Total number of bands =22		**17**	**19**	**13**	**16**	**17**	**16**	**19**	**20**

PEG= Polyethyleneglycol; MW= Molecular weight; 1= Presence of band; 0= Absence of band

Table 4. RAPD-PCR primer sequences and number of dominant RAPD bands of two flax varieties treated with different drought levels

Primer code no.	Primer sequences	Size range of the scorable loci (bp)	Total loci	No. of monomorphic loci	No. of polymorphic loci	% Polymorphism	Unique loci	Molecular size of markers (bp)
RAPD-1	GTTTCGCTCC	90-410	4	2	2	50	1	-330
RAPD-2	AACGCGCAAC	150-600	9	7	2	22.22	0	-
RAPD-3	CCCGTCAGCA	90-200	3	2	1	33.33	1	-200
RAPD-4	CCGGATCTAC	172-900	15	10	5	33.33	0	-
RAPD-5	AAGCCCGAGG	155-1400	16	10	6	37.50	1	+1302
RAPD-6	AAGGCGGCAG	121-850	11	6	5	45.45	3	+463, +610,+850
RAPD-7	GGACGGCGTT	145-990	17	7	10	58.82	5	+391, +680,-145, -788, -990
Total		**90-1400**	**75**	**44**	**31**	**41.33%**	**11**	**14.67%**

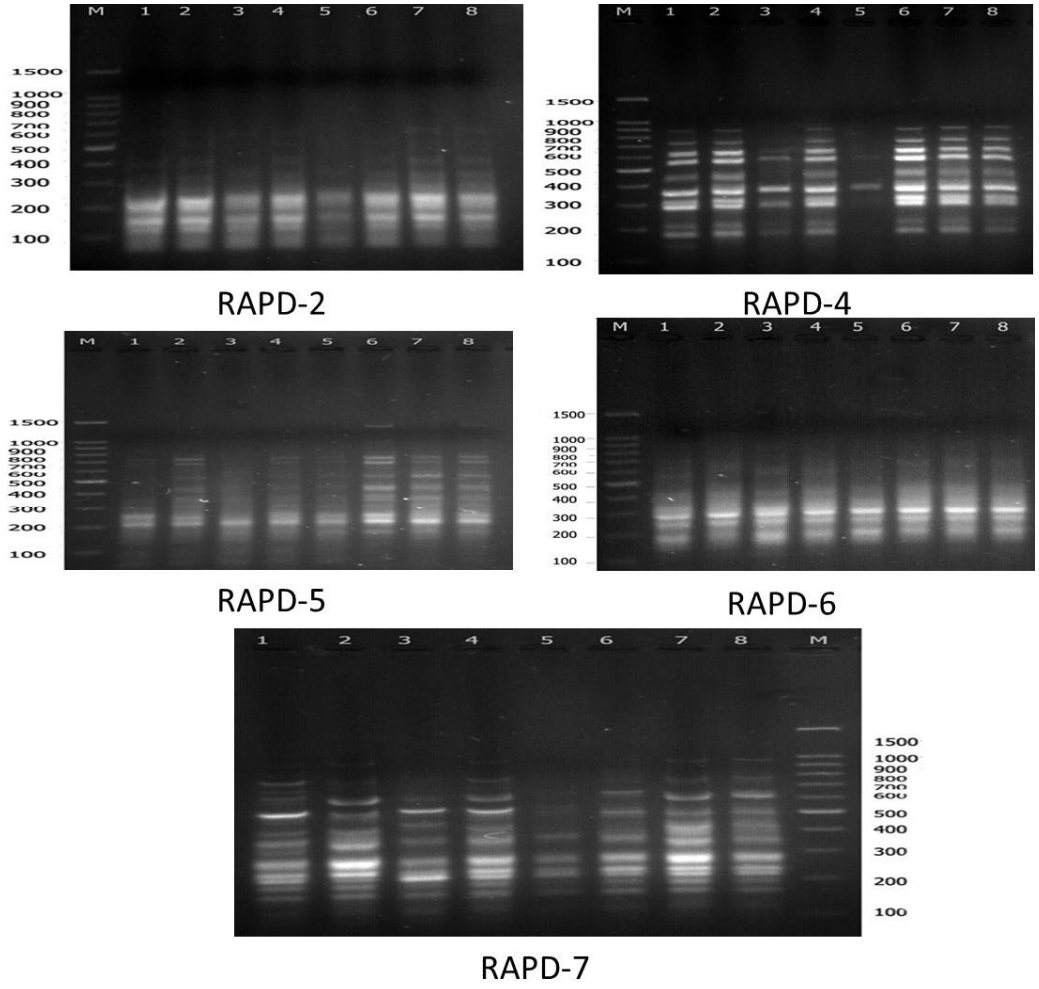

Fig. 4. Electrophoretic banding pattern amplified by RAPD-PCR marker in two flax varieties under drought stress compared with the control. Lane M= 100 bp DNA ladder.

Lanes 1= control of Asile variety, lanes 2, 3 and 4= represent 5, 10 and 15% PEG10,000, respectively) of Asile variety. Lane 5= control of Gentiana variety, lanes 6, 7 and 8 = represent 5, 10 and 15% PEG10,000, respectively) of Gentiana variety.

Addition to, three loci with molecular sizes +391, +1302 and +850 bp were recorded in the Asile control and plants treated with 5 and 10% PEG10,000, respectively. Furthermore, two negative markers of -200 and -330 bp were detected in the plants stressed with 10% PEG 10,000 and the control of Gentiana variety, respectively. Also, one allele of +680 bp revealed in 5% PEG10,000 for Gentiana variety using primer RAPD-7 (Table 4). There was a marked difference in the number of dominant RAPD-PCR fragments among the plants stressed at different drought levels and the control of both flax varieties. The polymorphism between flax varieties was 41.33%. Even the RAPD marker appeared various loci that were either present or absent within drought tolerant flax varieties. Identified polymorphic loci could be considered molecular markers to characterize drought tolerant varieties for the marker assisted selection (MAS) in flax breeding programs. These results showed similarity with those obtained by Liviero et al. [33] found that existence of more than one polymorphic band illustrate that drought tolerant cultivars differed from susceptible ones. The studies also show that, RAPD assay has a big potential to find DNA based polymorphism between the lines of same species. These identified polymorphic loci can be considered markers to characterize drought tolerant cultivars for MAS in wheat breeding programs. RAPD and ISSR techniques were

specifically screened as the amplified fragments are random by decamer primers. These primers link somewhere in the DNA sequence, finally assisting us at least in collecting data of trait linked regions. Deshmukh et al. [34] mentioned that molecular markers could be efficiently used to select and identify drought tolerant cultivars at an early stage. Thus, drought tolerance is controlled by a polygenic character; it needs identification which can be achieved by apply of molecular markers as they provide a faster method to identify drought tolerance associated areas. Huseynova et al. [35] stated that RAPD-PCR assays are a hopeful start for future investigations of water deficiency plant tolerance. Studies accomplished at molecular-genetic level allow appearance genetic markers for the selection of cultivars tolerant to adverse environmental conditions in a short time. El-Sayed and Rafudeen [36] found that markers linked with drought tolerance will help to identify of the genes included in lines. The sequence information could be applied to get PCR primers as diagnostics tools for drought tolerance.

4. CONCLUSIONS

Due to lack of irrigation water and increased evaporation from high temperature ascribed climate changes, breeding of flax tolerant varieties is a main aim in flax breeding programs in Egypt and worldwide. This study is the first report on the effect of water stress induced by large molecular weight *PEG 10000* on the flax. The results showed that Asile (the Indian) variety was the more tolerant to drought than Gentiana (the Romanian) variety which had the higher germination percentage. Also, this study not only appeared the meaning of identification of RAPD assay linked with drought tolerant loci but also defined polymorphism between untreated and treated flax varieties; while some advanced methodologies are needed to produce greater specificity of drought tolerant regions. The RAPD profiles have showed to be an easy, rapid and efficient method to identify such loci.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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