In vitro selection and genetic improvement of drought tolerance in canola (*Brassica napus*) using biochemical and molecular analyses

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Canola (*Brassica napus L.*) belongs to the genus *Brassica* from the family *Brassicaceae*, which is known to contain greatly diversified plant species. Canola, also called rapeseed, ranks only behind soybean and palm oil in global production, cultivated worldwide for oil production and considered as one of the essential sources for biodiesel fuel (Shokri-Gharelo and Noparvar, 2018). The term canola, abbreviation of Canadian Oil Low Acid. *B. napus* (AACC, 2n=38) according to (Nagaharu, 1935) is an allotetraploid species evolved hybridization between the diploid species *B. rapa* (AA, 2n=20) and *B. oleracea* (CC, 2n=18).

Canola oil is the most important for human consumption. It contains not more than 6 % of saturated fatty acids and about 96 % of its content is non-saturated fatty acids and very low level of low denesity lipids (Cholesterol) (Al-Naggar *et al*., 2008). Canola meal (remainder of canola seed after oil extraction) is the second-largest protein source after soybean meal for feeding animals, primarily for cattle and pigs.

Cultivation of canola in Egypt may provide an opportunity to overcome some of the local deficit of vegetable edible oil production, particularly it could be successfully grown during winter season in newly reclaimed land outside the old one of Nile valley; where clover and wheat, to get-around the competition with other crops occupied the old cultivated area. Suitability of growing canola under Egyptian conditions, compared with other oil crops, may be ascribed to its tolerance to harsh environmental influences frequently prevailing in such newly reclaimed soil such as salinity and drought (Megawer and Mahfouz, 2010).

Plant tissue culture refers to the *in vitro* culture plants from different parts on nutrient media under aseptic conditions (Salecjalali *et al*., 2011). The regeneration accompanied by the organogenesis has deployed various explants such as the cotyledon or hypocotyl segments (Mashayekhi *et al*., 2008). Somaclonal variation provides a valuable source of genetic variation for the improvement of crops through the selection of novel variants, which may show resistance to disease, improved quality, or higher yield (Ali, 1998; Ali and Metwally, 1992; Unai *et al*., 2004; Aboulila, 2016; Ali *et al.*, 2017).

Egypt. J. Genet. Cytol., 48:245-269, July, 2019
Web Site (*www.esg.net.eg*)

Drought tolerance is a relative term, which describes the ability of a plant to withstand a period of dryness from insufficient water supply. It is one of the major limiting factors and causes a severe reduction in agriculture crop production. Canola is generally considered to be more susceptible to drought. The yield is mainly affected by water shortages (Din *et al.*, 2011). Drought-induced significant decrease in metabolic factors such as the decrease in chlorophyll contents in canola plants (Hadi *et al*., 2014). Seed fatty acid composition, oil content and protein content of rapeseed are also affected by drought stress, according to Aslam *et al.*, (2009).

Genetic diversity in these plants has been characterized by using various morphological, protein, isozymes, and DNA-based markers. The success of any crop improvement program depends on the extent of genetic diversity available in the material (Shengwu *et al.*, 2003).

Isozymes are enzymes sharing a common substrate but differ electrophoretic mobility. Isozyme markers have certain advantages as isozyme loci are usually co-dominant and there is rarely any epistatic interaction (Market and Moller, 1959). It has been used as useful markers in genetic studies of many plant species. Isozyme loci have been used as markers in a number of *Brassica* genetic studies diversity in *B. juncea* (Kumar and Gupta, 1985) and in *B. rapa* (Persson *et al*., 2001). The relation between drought stress and SDS-PAGE protein may not reported before, but several research groups have reported variation in response to salinity. Those variations include the novel expression; overexpression and repression of some proteins (Youssef *et al*., 2006 and Al-Naggar *et al*., 2008). Molecular markers are the best tools for determining genetic relationships. A variety of molecular markers has been used to study the extent of genetic variation in two diverse groups of important crops in the genus *Brassica* (Liu *et al.*, 2006).

Random amplified polymorphic DNA (RAPD) markers offer a quick screening of different regions of the genome for genetic polymorphisms. This technique gained importance due to its simplicity, efficiency, relative ease to perform and non-requirement of sequence information (Karp *et al*., 1997). This study was designed to establish an efficient *in vitro* plant regeneration protocol in canola and induction of somaclonal variations under mannitol induced drought stress in Egypt. Characterization of the selected somaclonal variants using esterase, peroxidase, total soluble protein, and RAPD banding patterns were the other aim of this study.

MATERIALS AND METHODS

This study was carried out at Genetics Department Laboratories, Faculty of Agriculture, Kafrelsheikh University, Egypt, from 2013 through 2019.

The plant materials contained six canola genotypes namely (Serw-4, Pactol,

Sakha-1, Line-99, Line-162 and Line-123); kindly provided by the Oil Crops Res. section, Field Crops Research Institute, Agric. Res. center, Sakha, Kafrelsheikh, Egypt, were used in this study as shown in Table (1). Canola seeds were sterilized as followed by the tissue culture laboratory of the Department of Genetics for sterilizing the seeds.

In vitro tissue culture for plant regeneration protocol

The sterilized seeds were germinated in gars 250 ml (10 seeds per gar) containing 50 ml of MS (Murashige and Skoog, 1962) medium free hormone supplemented with 3% sucrose, 0.2% phytagel and the pH was adjusted to 5.8 before autoclaving. The cultures were incubated at 25±2°C under the darkness, for 3- 5 days and then completed at 25°C under a 16/ 8 light/ dark photoperiodic regime.

For callus induction hypocotyl segments (4 - 6 mm) excised from 7-day-old canola seedlings were used as explants in this study. They were cultured on six different pretreatment media for callus induction as shown in Table (2). All the media were adjusted to pH 5.5 and autoclaved at 121 °C for 20 min. and cultures were kept under 16 h photoperiod. After establishment, cultures were sub-cultured for 3-4 weeks intervals on fresh media.

After three weeks culture, the calli were sub-cultured onto medium containing 6mg/l BA and 2mg/l IBA for shoot regeneration and they were sub-cultured every three weeks on fresh medium for shoot multiplication and maintained at 25±2 °C under fluorescent light for 16 h photoperiod.

After six weeks of explant culture, callus induction percentage was calculated (number of explants produced callus/number of initiated explants x 100), regeneration percentage (the number of explants with shoots to the number of cultured explants x 100). The number of shoots per callus (number of regenerated shoots/ callus).

Selection of drought tolerance using mannitol treatment

After three weeks of explant culture, callus was cultured on media containing 6mg/l BA, 2mg/l IBA, 3% sucrose and 0.2% phytagel supplemented with three levels of mannitol (100, 150 and 200 mM) as well as medium free mannitol as control and the pH was adjusted to 5.5 before of autoclaving. Cultures were kept under the defined temperature (25±2 °C) and lighting condition (16 h photoperiod) and the cultuvability traits were calculated.

Statistical analysis

The data were analyzed by ANOVA (analysis of variance) to test the differences between the performances of genotypes and different media for each genotype (Snedecor, 1962).

Biochemical and molecular assessment of somaclonal variations

Biochemical analysis using isozymes (esterase and peroxidase) and total protein was carried out to determine somaclonal variations among the parental genotypes and regenerated derived plants which have been developed on different culture media.

Samples (extraction of green parts) were prepared by weighting 0.5 gm fresh leaves from regenerated plants as well as parental genotypes. Each sample was homogenized in 1.0 ml ice-cold 0.02 M tris-HCl buffer (pH 6.8) containing 20 % w/v sucrose, 5 mM dithiotritol (DTT), 0.03% (v/v) β-mercaptoethanol and 4 % (w/v) polyvinyl-polypyrrolidone. The extracts were then centrifuged at 12,000 rpm at 4 °C for 15 min. and the supernatant was pipette.

Peroxidase and easterase detection on gel

Both peroxidase and easterase bands were detected as described by (Scandalios, 1964 and 1969).

SDS-polyacrylamid gel electrophoresis

Total protein was separated using SDS-PAGE according to Laemmli, (1970) using 12.5 % acrylamide in the separating gel and 4 % in the stacking gel.

Isolation of genomic DNA and amplification conditions for RAPD

DNA isolation was performed using Cetyl trimethyl ammonium bromide (CTAB) based procedure according to (Murray and Thompson, 1980) and the DNA was quantified on a 1.5 % agarose gel using standard DNA ladder. Five ten-mer of random primers (OPA-09, OPA-20, OPB-05, OPB-06 and OPB-07) was used as shown in Table (3). Polymerase Chain Reaction (PCR) amplification and the DNA amplified fragments were done according to Aboulila (2016). Bands were scored as 1 if present or 0 if absent.

RESULTS AND DISCUSSION

Tissue culture traits in normal condition

The results showed that there were a highly and significantion viewpoint of callus induction, callus fresh weight, callus size and the number of shoots per callus among cultivars as shown in Table (4). For callus induction percentage (%) the results revealed that callus was formed from the used explants of the six genotypes on all tested concentrations of plant growth regulators as illustrated in Table (5) and (Fig.1). The percentages of callus formation C% varied according to both of the used genotype and the concentration of growth hormones. For all *B. napus* cultivars, higher percentages of callus formed ranging from 46 to 100 % on the different media Table (5). Moreover, among the six tested media for callus induction, MS_6 medium (MS + 0.1 mg/l NAA + 0.5 mg/l

Kin) gave the best results on average, with 100% for all tested genotypes, except line-162 was 94%. On the other hand, MS_5 medium (MS+ 0.5 mg/l BA+ 0.5 mg/l Kin) gave the lowest mean value (63.67%) for all tested genotypes (Table 5). Regarding genotypes, Sakha-1 had the highest mean value (100 %) for callus induction on MS_3, MS_4 and MS_6 and 99% on MS_1, while the minimum rate (78 %) was recorded on MS_5. Mean percentage of callus induction was 96% for Sakha-1, followed by Line-123 (94.19%), Line-99 (93.16%), Pactol (83.16%) and Serw4 (78.81%). The lowest responses were noted for Line 162 (74.65%).

For mean fresh weight of callus (gm) the genotype Line-99 exhibited the highest fresh weight mean value (0.326 gm), followed by Serw-4 (0.259 gm), while the lowest value (0.162 gm) was recorded for Pactol genotype Table (6). Meanwhile, MS_6 gave the highest fresh weight mean value (0.337 gm), followed by the values of MS_3 (0.279 gm), while MS_2 gave the lowest value (0.175 gm) for all tested genotypes.

Concerning callus size (cm^3) the genotypes Line-99 exhibited the highest callus size value ($0.432cm^3$), followed by Line-123 (0.401 cm3), while the lowest value ($0.244cm^3$) was recorded for Line-162. Meanwhile, MS_4 medium gave the highest value (0.525 cm^3), followed by the mean value of MS_6 medium (0.416 cm3), while MS_2 gave the lowest mean value (0.168 cm^3) Table (7).

For shoot induction percentage on regeneration media the results revealed highly significant differences between the genotypes on regeneration media. Concerning genotypes, Sakha-1 exhibited the highest value (58.84%) for regeneration rate, then Line-99 (58.46%) and Serw-4 (49.3%). The lowest response was Pactol (42.6) Table (8).

The mean number of shoots per callus no significant differences were found between line-99 and line-123 genotypes, but there were significant differences between Serw-4, Pactol and Sakha-1. Also, Sakha-1 gave the highest mean number of shoot/callus (7) while the lowest number was recorded by Serw-4 genotypes (2.47 shoot/ callus) as shown in Table (8).

In the present protocol, hypocotyl explants resulted in a higher percentage of callus formation, ranging from 63.7 to 99.4% on different media. (Burbulis *et al*., 2009) reported that the high efficiency of stem regeneration depends on the type of explants used and genotype. Moreover, among six tested media, MS_6 (MS + 0.1 mg/l NAA + 0.5 mg/l Kin) gave the best results on average of callus induction percentage (99.4%) and then MS_4 (MS + 1.0 mg/l BA + 1.0 mg/l NAA) (97.4%). The results showed that the MS_1 medium was the best media for the regeneration rate and the number of shoot/callus. These results agree with that of Chamandoosti

and Azad, 2012 who studied modified methods for callus induction and plant regeneration in canola to produce resistant canola by somaclonal variation against the sclerontinia stem root which caused by the pathogen *Sclerontinia sclerotoiorum* and they showed that the best regeneration media was MS_1.

In this study, the age of explants was (7 days) and pH of media was 5.5 and both are important factors for organogenesis and regeneration of plantlets in canola according to (Smith and Krikorian, 1990; Chamandoosti and Azad, 2012). The use of hypocotyls and/or cotyledons as explants for *in vitro* plant ratio regeneration has received significant attention. Moreover, as many studies indicated, these explants possess a high capacity for shoot organogenesis, somatic embryogenesis and protoplast culture (Gerszberg *et al*., 2015).

Tissue culture under drought stress induced by mannitol

The selection program described in materials and methods section is easy to perform and allows selection during callus regeneration on media containing 6 mg/l BA, 2 mg/l IBA and increasing mannitol levels (0.0, 100, 150 and 200 Mm).

The obtained data indicated that the better response for drought stress was recorded by Line-99 then Serw-4 for callus weight, callus size and the number of shoots per callus. Data of callus weight, callus size and the number of shoot per callus are presented in Table (9) and (Fig. 2). The data showed that Line-99 had a better response than Serw-4 on free mannitol (control) medium or media under drought stress. The highest callus weight (0.45 g) was achieved at free mannitol (control media). For the callus under stress, the highest weight (0.28 gm) was observed on Line-99 under 100 mM mannitol, and on the same condition, the number of shoots was 3.3.

The data showed also that all studied genotypes were susceptible to drought stress but both Line-99 and Serw-4 were the best-responded ones. In this respect, similar results were previously recorded by (Din *et al*., 2011). Drought stress decreased the weight and size of callus (Massonnet *et al.,* 2007; Zhang *et al.,* 2014) they reported that the degree of effect drought effect on seed germination, plant growth, flowering, seed yield and quality depends on the plant physiological, biochemical and molecular processes, as well as the ability of the plant to adapt to drought stress.

Biochemical analyses

For peroxidase isozyme patterns, the results in Table 10 and Fig. (3A) revealed that both origin genotypes (Sakha-1 and Line-123) had the same two bands (n.3 and n. 5) which they were different with their derivative ten somaclones in the number (ranged between seven bands in the somaclone SV-1 and one band in somaclones SV-2, SV-5, and SV-7) and in the intensity of

bands (all regenerated plant from Sakha-1 variety). Meanwhile, the three somaclones regenerated from Line- 123 had the same number of bands (five bands) two of them (SV-8 and SV-9) were similar in isozyme pattern. On the other hand, there were some differences in the activity of bands between the original genotypes and their derivative somaclones, as well as between somaclones each other. In addition, the n.5 was present in all tested genotypes (monomorphic) while the rest six bands were polymorphic bands with 85.7 % polymorphism. Out of the six polymorphic bands, one was a unique band for somaclone variant SV-1.

Regarding esterase isozymes, the isozyme patterns of the two original genotypes (Sakha1 and Line-123) and the ten regenerated plants Table (11) and Fig. 3B) yielded 17 bands. Most of these bands (16 bands) were polymorphic with 94.1% polymorphism and differed in both number and in intensity. The two origin genotypes had the same number of bands (eight bands), while the number of bands in their derivative somaclones number ranged between eleven bands in two both SV-1 and SV-8 and two bands in SV-10. One monomorphic band (no.3) was detected in all of the regenerated plants with their two parents. Out of the 16 polymorphic bands, one was a unique band (no.14) for somaclone variant SV-1 related to Sakha-1 cultivar.

Concerning the peroxidase isozymes under drought stress, two origin genotypes (Serw-4 cultivar and Line-99) and five somaclones yielded seven bands ranged between seven bands in SV-d stressed somaclone and one band was given by the origin Line-99 (Table 12 and Fig. 4A). While six bands were polymorphic with 85.7% polymorphism and differed in both number and in intensity as well as one monomorphic band (no.5) were detected. On the other hand, two unique bands were detected (nos.6 and 7) related to tolerated SV-d somaclone derived from Serw-4 cultivar (may be used as markers for Serw-4 derived somaclones).

For description of esterase patterns of canola under drought stress, results revealed that two origin genotypes (Serw-4 cultivar and Line-99) and five somaclones yielded seven bands ranged between seven bands in tolerated SV-d somaclone and 0 bands was found by the SV-d1 stressed somaclone related to the origin Line-99 (Table 13 and Fig. 4B). All detected bands exhibited 100% polymorphism. No unique bands were detected in these patterns.

Regarding the total protein analysis, the results in Table (14) and Fig. (5) showed a total number of 26 bands which were distributed in all genotypes with molecular weights ranged from 10.96 as

monomorphic band to 224.17 KDa found in both SV-7 somaclone related to its origin Sakha-1 and origin Line-123. The percentage of polymorphism was 80.77%. All studied genotypes were different identifiable as shown in Table (14). Protein pattern showed that almost of bands were differ in their intensity among the studied genotypes. Furthermore, some genotypes possessed some bands which were absent in their parent such as bands number 1, 2, 3, 4, 6, 13, 18, 20 and 21 in Sakha-1 and bands number 12, 13, 22 and 23 in Line-123, these bands may be related to drought tolerance in canola. Moreover, four bands were detected as unique bands (no. 3, 12, 18, and 23 with molecular weights of 163.19, 64.23, 28.51, 26.41 and 16.21 KDa) in somaclones nos. SV-7, SV-10, SV-7, and SV-9 beside the band no. 19 related to origin genotype Line-123. In addition to five bands detected as monomorphic bands (nos. 5, 7, 10, 14 and 26).

A comparative study of peroxidase and esterase isozymes and total soluble protein analyses was carried out to establish an efficient *in vitro* plant regeneration protocol in six canola genotypes and induction of somaclonal variation under control and three stress drought levels of mannitol media in order to characterize and evaluate derived canola somaclones. The results showed a varying number of isozyme bands for both isozymes. The appearance and disappearance of bands, along with their intensity scores, indicated the role of different isozymes affected by *in vitro* and tissue culture. Somaclonal variants in plants produced via tissue culture can be used to generate new cultivars (somaclones) (Muller *et al.*, 1990). Plant cell and tissue culture itself generates somaclonal variation and produced genetically changed plants. At genetic level, somaclonal variation can be brought about by various DNA changes. Larkin and Scowcroft, 1981 and Evans, 1989 stated that somaclonal variation provides an opportunity for crop improvement.

Assessment of genetic diversity using RAPD analysis

DNA profiling with suitable RAPD primers could be used for identification and discrimination among oilseed rape cultivars (Mailar *et al.,* 1997; Ahmed *et al.*, 2007). Therefore, this technique was applied to measure the degree of genetic variability among the 19 selected canola genotypes. DNA from 19 canola genotypes (four parental genotypes and 15 somaclonal variants, three out of them were generated under drought stress) was amplified with five random 10-mer primers as shown in Fig. (6) and Table (15). The percentage of polymorphism ranged from 66.67 % for OPB-07 to 91.67% for OPA-20. A total of 94 amplicons (ampli-

fied fragment) were generated by all primers with sizes ranged from 169 bp to 2211 bp. These primers gave 78 polymorphic bands, out of them 18 were unique which could be used as genetic fingerprints for those genotypes. Ten out of 18 obtained positive unique bands were appeared in the drought tolerant somaclonal variants. These bands may play a positive role in the drought tolerance process.

These findings further strengthened previous reports by (Abbas *et al.,* 2009), who mentioned that the RAPD markers could be used effectively to estimate genetic diversity among canola genotypes. Moreover, (Afia *et al.,* 2007) found that only four primers out of six developed ten molecular markers under stress tolerance, four of them were positive; while the other six were negative ones. These primers generated 19 DNA fragments, their sizes ranged from 271 to 2211 bp.

Sum of PIC value for all alleles of each primer was in the range of 0.183 to 0.30. According to the obtained results (Table 15), primer OPA-20 was distinguished as the best primer for genetic diversity analysis. Many authors have successfully used RAPD technique for assessment of genetic diversity and inferred that PCR based assays can be effectively used to analyze the genetic diversity in *B. napus* (Ebrahimi *et al.,* 2011; Abdelmigid, 2012; Fahmi *et al*., 2012).

SUMMARY

Callus induction methods and plant regeneration system in six selected canola (*Brassica napus*) cultivars“Serw-4, Pactol, Sakha-1, Line-99, Line-162 and Line-123" grown under Egyptian environment were investigated for drought stress. Biochemical and molecular characterization were the other goal for this study. Hypocotyl explants (5-7 mm) from sterile canola seedling were cultured on MS media with different concentrations of plant growth regulators. The results of callus induction varied with respect to treatments and genotypes. For *B. napus*, the use of hypocotyl explants resulted in a higher percentage of callus formation, ranging from 46 to 100% on different media. Moreover, among six tested media, MS_6 (MS + 0.1 mg/l NAA + 0.5 mg/l Kin) gave the best results on average of callus induction percentage with 100% of explants producing callus for cv. Serw-4, Pactol, Sakha-1, Line-99 and Lin-123. Meanwhile, Line-162 recorded 94% for callus induction. The lowest responses were noted for MS medium with 0.5 mg/l BAP + 0.5 mg/l Kin (MS_5). Sakha-1 was the most responsive in terms of percentage of callus induction, while recorded 100 % on MS_1, MS_3, MS_4 and MS_6, 96 % on MS_2 and 78 % on MS_5. As well as the highest percentage of explants with shoot induction (58.84 %) and the highest number of shoot per explant (7) was recorded for this genotype. Assessment of isozyme banding patterns (esterase and peroxidase) to detect more genetic markers was also another attempt for this study. A total of 94 amplified fragments were generated by

five random 10-mer primers, 78 polymorphic bands were detected in RAPD analysis, out of them, 18 were unique bands. These unique bands can be used as a specific marker for drought tolerance in canola.

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Table (1): Name and origin of the parental canola genotypes used in this study.

No.	Genotype	Name	Origin
1	Serw-4	Serw-4	Egypt
2	Pactol	Pactol	France
3	Sakha-1	Sakha-1	Egypt
4	Line-99	Niklas	FAO
5	Line-162	125-001	Austra
6	Line-123	Wuhan	FAO

Table (2): Growth regulators content used in each experiments for callus induction.

Pre-treatment media	Hormone Combination	References
MS_1	BA (6mg/l) + IBA (2 m/l)	Chamandoosti and Azad, (2012)
MS_2	BA (3mg/l)	Terinejod (2013)
MS_3	NAA (0.1 mg/l)	Terinejod (2013)
MS_4	BA (1mg/l) + NAA (1mg/l)	---
MS_5	BA (0.5 mg/l) + Kin (0.5mg/l)	---
MS_6	NAA (0.1mg/l) + Kin (0.5 mg/l)	Terinejod (2013)

Table (3): Names and nucleotide sequences of the used RAPD primers.

Primer name	Primer sequence (5′→ 3′)
OPA-09	GGGTAACGCC
OPA-20	GTTGCGATCC
OPB-05	TGCGCCCTTC
OPB-06	TGCTCTGCCC
OPB-O7	GGTGACGCAG

Table (4): Mean squares (MS) for the percentage of callus formation (C %), callus fresh weight, callus size and the number of shoots per callus for canola genotypes cultured on six different culture media.

Source of variation	D.F.	C %	Callus fresh weight (gm)	Callus size (cm^3)	Shoot/callus
Genotypes (G)	5	1452.2**	0.054308**	0.1339**	110.92**
Media (M)	5	3022.8**	0.0644752**	0.33351**	608.74**
REB (B)	2	273.4*	0.024752ns	0.027910ns	25.095**
G*M	25	180.17**	0.041892**	0.05799**	46.262**
G*B*M	70	63.048	0.011758	0.019679	7.3288

**, *, ns indicate significant differences at P<0.01, P<0.05 and not significant, respectively, according to F test.

Table (5): Mean percentage of callus induction under the different media for six canola genotypes

Genotypes	MS media						Mean
	MS_1	MS_2	MS_3	MS_4	MS_5	MS_6	
Serw-4	68	67b	74	98	56	100	78.81
Pactol	88	77.5	40	98	54	100	83.16
Sakha-1	99	96	100[a]	100	78	100	96.08
Line-99	98	96	100	100	66	100	93.16
Line-162	62	62	84	84	46	94	74.65
Line-123	99	97	98	100	72	100	94.19
Mean	86.26	82.62	90.50	97.44	63.67	99.44	

$L.S.D._{0.05} = 1.994$

Table (6): The mean fresh weight of callus for six genotypes on six different media.

Genotypes	MS media						Mean
	MS_1	MS_2	MS_3	MS_4	MS_5	MS_6	
Serw-4	0.13	0.44	0.99	0.166	0.26	0.58	0.259
Pactol	0.102	0.056	0.059	0.14	0.097	0.047	0.162
Sakha-1	0.16	0.015	0.234	0.174	0.219	0.175	0.255
Line-99	0.188	0.119	0.53	0.353	0.156	0.218	0.326
Line-162	.064	0.039	0.104	0.103	0.084	0.257	0.210
Line-123	0.24	0.413	0.207	0.158	0.132	0.171	0.246
Mean	0.215	0.175	0.279	0.237	0.193	0.337	

Table (7): The mean size of callus trait for the six canola genotypes (Serw-4, Pactol, Sakha-1, Line-99, Line-123 and Line-162) using hypo-cotyl as explant on six different culture media.

Genotypes	MS media						Mean
	MS_1	MS_2	MS_3	MS_4	MS_5	MS_6	
Serw-4	0.1167	0.10	0.2667	0.45	0.3633	0.45	0.291
Pactol	0.2167	0.1333	0.2833	0.45	0.3153	0.2733	0.246
Sakha-1	0.225	0.2833	0.4833	0.2667	0.3417	0.4687	0.345
Line-99	0.25	0.2083	0.2933	0.8667	0.2667	0.7067	0.432
Line-162	0.1167	0.0833	0.2667	0.2833	0.2667	0.45	0.244
Line-123	0.1967	0.20	0.4950	0.833	0.3333	0.3467	0.401
Mean	0.1869	0.1681	0.3481	0.5250	0.3145	0.4167	

$L.S.D._{0.05}$ =1.994

Table (8): Shoot induction percentage, the mean number of shoots per callus and the percentage of rooting (%) for all studied genotypes.

Genotypes	**Serw-4**	**Pactol**	**Sakha-1**	**Line-99**	**Line-123**	**$LSD_{0.05}$**
Regeneration %	49.380	42.620	58.840	58.460	58.50	2.014
No. shoots/callus	2.4737	3.2105	7.0	3.8947	3.736	1.987

Table (9): Response of callus weight, callus size and number of shoots per callus to mannitol drought stress.

Mannitol (mM)	Callus fresh weight (gm)		Callus size (cm^3)		Shoot/callus	
	Serw-4	Line-99	Serw-4	Line-99	Serw-4	Line-99
0.0 (1)	0.2736	0.4520	0.7238	0.8550	3.2	3.8
100 (2)	0.1531	0.2837	0.5106	0.6275	2.9	3.3
150 (3)	0.1161	0.1704	0.5098	0.5600	2.6	2.8
200 (4)	0.1067	0.1347	0.4634	0.5438	1.5	1.8

$LSD_{0.05}$= 2.12

Table (10): Discription of peroxidase patterns of canola

No. band	RF	Sakha-1 (c)	SV-1	SV-2	SV-3	SV-4	SV-5	SV-6	SV-7	Line-123 (c)	SV-8	SV-9	SV-10	Polymorphism
1	0.061	-	+++ +	-	+	++	-	-	-	-	+	++	++	Polymorphic
2	0.121	-	+	-	+	+	-	-	-	-	-	-	+	Polymorphic
3	0.226	+	+	-	+++	++ +++	-	+++ ++	-	+++	+++ +++	++	+	Polymorphic
4	0.350	-	+	-	-	+	-	-	-	-	+	+	-	Polymorphic
5	0.498	+++	+++ + +++ +	+++ +++ +	+++ + +++	+++ +++ +	++++ ++++ +	+++ ++++ +	+++ +++ +	++++	+++ +++ +	+++ + +++ +	++++ ++++	Monomorphic
6	0.697	-	++	-	-	-	-	-	-	-	-	-	-	Unique
7	0.845	-	+	-	-	-	-	-	-	-	+	+	+	Polymorphic
Total		2	7	1	4	5	1	2	1	2	5	5	5	85.714%

- absent + very weak ++ weak +++ intermediate ++++ dark +++++ very dark

Table (11): Discription of esterase patterns of canola.

No. bands	RF	Sakha-1 (c)	SV-1	SV-2	SV-3	SV-4	SV-5	SV-6	SV-7	Line-123 (c)	SV-8	SV-9	SV-10	Polymorphism
1	0.048	+	+	+	-	+	+	-	+	+	+	+	+	Polymorphic
2	0.093	-	-	-	-	-	-	-	-	++	+	-	-	Polymorphic
3	0.117	+++	++	+	+	+	+	+	+	++	++	++	++	Monomorphic
4	0.171	++	+	-	+	+	+	+	+	-	+	+	-	Polymorphic
5	0.407	-	-	+++	-	-	-	-	-	+	-	-	-	Polymorphic
6	0.462	++	++	-	-	-	-	-	-	-	-	-	-	Polymorphic
7	0.343	++	++	+	+	-	+	+	+	++	-	++	-	Polymorphic
8	0.289	++	+++	-	-	-	-	-	++	++	++	++	-	Polymorphic
9	0.204	-	++	-	-	-	-	-	-	++	++	++	-	Polymorphic
10	0.241	+++	+++	+++	+++	++++	-	+++	+++	-	+++	+	-	Polymorphic
11	0.530	-	+	-	++++	+++	-	+	-	-	-	+	-	Polymorphic
12	0.628	+++	+++	+++	+++	+++	-	+++	-	-	+++	+++	-	Polymorphic
13	0.692	-	-	-	-	-	-	++	+	-	-	-	-	Polymorphic
14	0.741	-	-	-	-	-	-	-	-	-	-	+	-	Unique
15	0.819	-	-	-	+	+	-	+	-	-	+	+	-	Polymorphic
16	0.960	-	-	-	-	-	-	-	-	-	++	++	-	Polymorphic
17	0.907	-	+	-	-	-	-	+	-	+	+	+	-	Polymorphic
Total		8	1 1	6	7	7	4	9	7	8	11	13	2	94.118%

- absent + very weak ++ weak +++ intermediate ++++ dark

Table (12): Discription of perxidase patterns of canola under drought stress imposed by mannitol.

No. band	**RF**	**Serw-4 (c)**	**SV-1**	**SV-d**	**Line-99 (c)**	**SV-1**	**SV-d1**	**SV-d2**	**Polymorphism**
1	0.030	-	-	++	-	++	-	+	Polymorphic
2	0.070	-	-	++	-	++	-	-	Polymorphic
3	0.125	-	-	++	-	++	-	-	Polymorphic
4	0.225	+	++	++++ +++++	-	++++ +	+++	++++	Polymorphic
5	0.494	+++	+++ ++++	+++++ ++++	++	+++++ +++++	+++ +++	++++ ++++	Monomorphic
6	0.625	-	-	++	-	-	-	-	Unique
7	0.722	-	-	++	-	-	-	-	Unique
Total		2	2	7	1	5	2	3	85.71%

- absent + very weak ++ weak +++ intermediate ++++ dark +++++ very dark

Table (13): Discription of esterase patterns of canola under drought stress imposed by mannitol.

No. band	RF	Serw-4 (c)	SV-1	Drought	Line-99 (c)	SV-1	SV-d1	SV-d2	Polymorphism
1	0.038	-	-	+	+++	+	-	-	Polymorphic
2	0.071	-	+	+	+++	++	-	+	Polymorphic
3	0.154	+	+	+	++	++	-	+	Polymorphic
4	0.206	-	-	+	+	++	-	-	Polymorphic
5	0.277	++	-	+	-	+++	-	-	Polymorphic
6	0.371	+++	+	+	-	+++	-	+++ +++	Polymorphic
7	0.463	+	++	+++	-	+++	-	+	Polymorphic
Total		4	4	7	4	7	0	4	100.000%

- absent + very weak ++ weak +++ intermediate ++++ dark

Table (14): Discription of total soluble proteins of the selected canola genotypes (Sakha-1 and Line-123) and their regenerated somaclones.

NO.	MW	Sakha-1	SV-1	SV-2	SV-3	SV-4	SV-5	SV-6	SV-7	Line-123	SV-8	SV-9	SV-10	Frequency	Polymorphism
1	224.17	-	-	-	-	-	-	-	+	+	-	-	-	0.167	Polymorphic
2	178.60	-	-	-	-	-	-	-	+	+	+	-	-	0.250	Polymorphic
3	163.19	-	-	-	-	-	-	-	+	-	-	-	-	0.083	Unique
4	138.08	-	-	-	-	-	-	-	++	++	-	-	-	0.167	Polymorphic
5	124.49	+	+	+	+	+	+	+	++	++	++	++	++	1.000	Monomorphic
6	112.24	-	-	+	+	+	-	-	++	++	+	+	+	0.667	Polymorphic
7	103.59	+	+	++	++	++	++	+	++	++	++	++	+	1.000	Monomorphic
8	93.71	+	+	+	-	++	+	-	+	++	+	+	+	0.833	Polymorphic
9	85.62	+	-	-	-	-	-	-	++	+	+	+	+	0.500	Polymorphic
10	78.23	+	+	+	+	++	+++	+	++	+++	++	++	+	1.000	Monomorphic
11	67.09	++++	++	++	-	-	-	-	++	++++	++	++	-	0.583	Polymorphic
12	64.23	-	-	-	-	-	-	-	-	-	-	-	++	0.083	Unique
13	54.35	-	-	+	-	-	-	-	+++	-	+	++	+++	0.417	Polymorphic
14	49.49	++++ +++	+++	+++	+++	++++	++++	+++	+++	++++ ++++	++++ +++	+++	++	1.000	Monomorphic
15	42.02	+++	-	+++	+++	-	-	-	+++	+++	++	++	++	0.667	Polymorphic
16	36.88	++	-	-	-	-	-	-	-	++	+	+	-	0.333	Polymorphic
17	32.05	++	-	++	-	-	-	+	++	++	++	++	++	0.667	Polymorphic
18	28.51	-	-	-	-	-	-	-	++	-	-	-	-	0.083	Unique
19	26.41	-	-	-	-	-	-	-	-	++	-	-	-	0.083	Unique
20	23.57	-	-	-	-	++++	-	-	++	++	+	++++	++++	0.500	Polymorphic
21	21.32	-	++++	+++	-	-	-	-	++	++	+	-	+	0.500	Polymorphic
22	18.59	++	-	-	-	-	-	-	++	-	-	++	-	0.250	Polymorphic
23	16.21	-	-	-	-	-	-	-	-	-	-	++	-	0.083	Unique
24	14.37	+	-	-	-	++++	+	-	++	+++	++	++	+	0.667	Polymorphic
25	12.16	++	-	-	-	-	-	-	+++	++	+	+	-	0.417	Polymorphic
26	10.96	++	+	+	+	++	+	+	+	+	+	+	+	1.000	Monomorphic
Total		14	8	12	7	8	8	6	22	20	18	18	15		

Table (15): RAPD analysis in canola genotypes under study.

Primer	OPA-09				OPA-20				OPB-05				OPB-06				OPB-07			
Genotype	M	P	Positive Unique	Total	M	P	Positive Unique	Total	M	P	Positive Unique	Total	M	P	Positive Unique	Total	M	P	Positive Unique	Total
1- Sakha-1 (Control)	3	6	0	9	1	6	0	7	2	7	0	9	4	6	0	10	6	5	0	11
2- Sakha-1 SV-1	3	5	0	8	1	7	0	8	2	7	0	9	4	8	0	12	6	6	0	12
3- Sakha-1 SV-2	3	5	0	8	1	8	0	9	2	10	0	12	4	5	1	9	6	6	0	12
4- Sakha-1 SV-3	3	4	0	7	1	7	0	8	2	6	0	8	4	7	0	11	6	4	0	10
5- Sakha-1 SV-4	3	9	0	12	1	6	0	7	2	7	0	9	4	6	0	10	6	5	0	11
6- Sakha-1 SV-5	3	6	0	9	1	7	0	8	2	7	0	9	4	7	0	11	6	7	0	13
7- Sakha-1 SV-6	3	9	0	12	1	9	0	10	2	7	0	9	4	7	0	11	6	3	0	9
8- Sakha-1 SV-7	3	6	0	9	1	7	0	8	2	10	0	12	4	6	0	10	6	6	0	12
9- Sakha-1 SV-8	3	8	0	11	1	7	0	8	2	8	0	10	4	4	0	8	6	3	0	9
10- Sakha-1 SV-9	3	9	0	12	1	8	0	9	2	7	0	9	4	4	0	8	6	6	0	12
11- Sakha-1 SV-10	3	8	0	11	1	5	0	6	2	7	0	9	4	8	4	12	6	3	0	9
12-Pactol (Control)	3	10	0	13	1	7	0	8	2	10	0	12	4	7	0	11	6	7	0	13
13- Pactol SV-11	3	10	0	13	1	6	0	7	2	8	0	10	4	4	0	8	6	5	0	11
14- Serw-4 (Control)	3	4	0	7	1	7	0	8	2	6	0	8	4	5	0	9	6	7	0	13
15- Serw-4 SV-12	3	10	0	13	1	5	0	6	2	9	0	11	4	6	0	10	6	7	0	13
16- Serw-4 SV-13D	3	2	0	5	1	2	0	3	2	8	1	10	4	6	1	10	6	6	0	12
17- Line-99 (Control)	3	5	0	8	1	3	0	4	2	8	2	10	4	7	0	11	6	8	1	14
18- Line-99 SV-14D	3	10	0	13	1	4	1	5	2	8	4	10	4	4	0	8	6	6	0	12
19- Line-99 SV-15D	3	7	3	10	1	2	0	3	2	7	0	9	4	7	0	11	6	6	0	12
Amplified bands	3	13	3	19	1	10	1	12	2	12	7	21	4	14	6	24	6	11	1	18
Polymorphism %	84.21 %				91.67 %				90.48 %				83.33 %				66.67 %			

Size range (bp)	271-2211	169-1184	189-1927	196-2164	274-1584
PIC	0.27	0.30	0.183	0.183	0.21

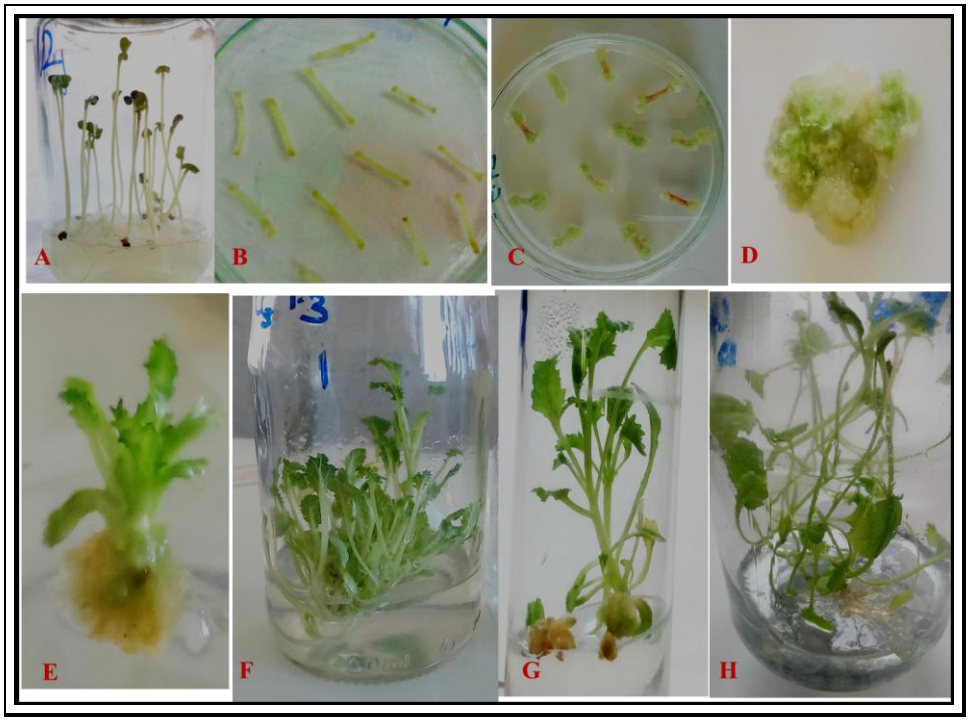

Fig. (1): A Seedlings cultured on MS basic medium, B. hypocotyls cultured on induction media, C. Callus induction on medium, D. Callus subculture, E. Shoot induction, F. shoot multiplication on regeneration medium MS with 6 mg/l BA and1mg/l IBA, G. Shoot elongation and H. Regenerated plants domesticated for rooting.

Fig.(2): Serw-4 (A) and Line-99 (B) under drought stress

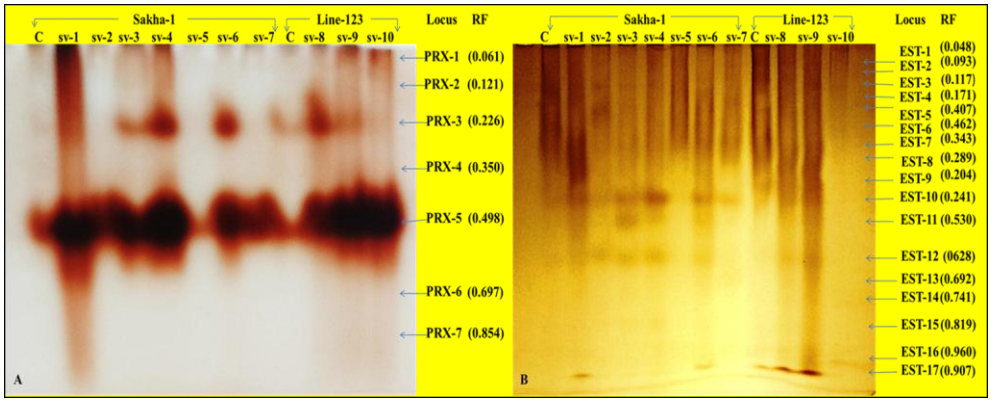

Fig.(3): Discription of peroxidase (A) and esterase (B) patterns of the tested canola genotypes.

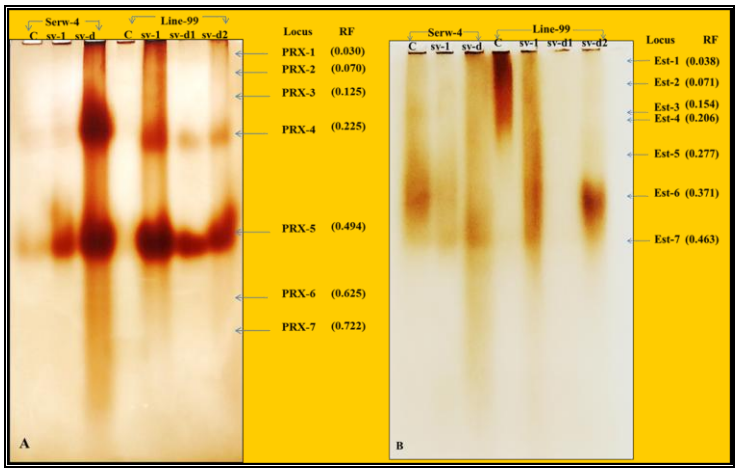

Fig. (4) Discription of peroxidase (A) and esterase (B) patterns of canola under drought stress imposed by mannitol.

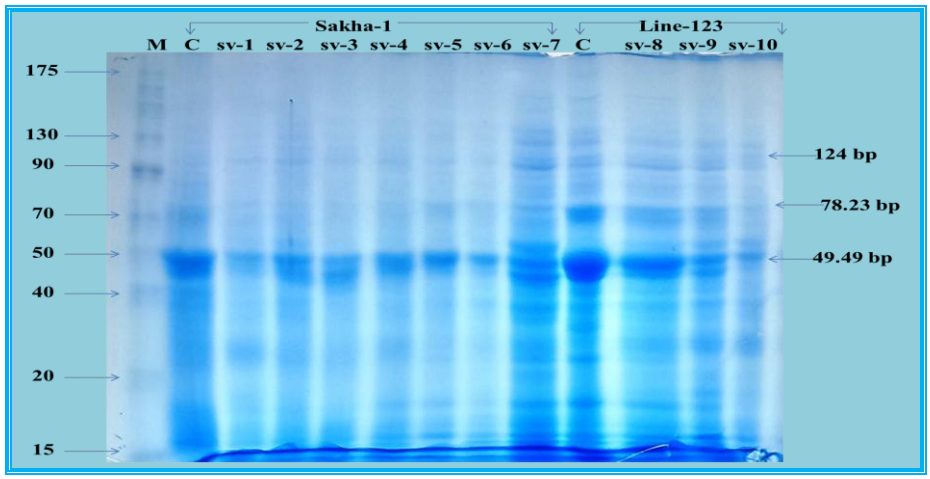

Fig. (5): SDS-PAGE of toal protein extracted from Sakha-1 and Line- 123.

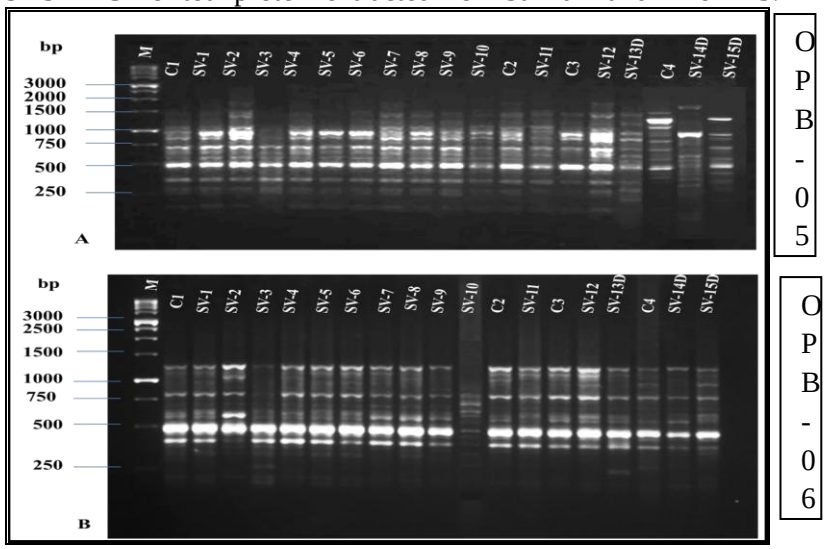

Fig. (6): RAPD fingerprints for OPB-05 (A) and OPB-06 (B) of the 19 selected canola genotypes as listed in Table (15).