JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4373-4384

In vitro Selection and Characterization of Drought-tolerant of some Fig (*Ficus carica* L.) Plants using DNA Markers

Ehab M.R. Metwali[1,2], Hemaid I.A. Soliman[3], S.M. Howladar[4], H.S. Al-Zahrani[1] and M.P. Fuller[5]

[1]Biological Science Department, Faculty of Science,King Abdulaziz University, 21589 Jeddah, Saudi Arabia.
[2]Botany Department, Faculty of Agriculture, Suez Canal University, 41522 Ismailia, Egypt.
[3]Plant Genetic Recourses Department, Desert Research Center,El-Matariya 11753, Cairo, Egypt.
[4]Biology Department, Faculty of Science, Albaha University, Albaha, Saudi Arabia.
[5]School of Biological Sciences, Faculty of Science and Environment, Plymouth University, Plymouth, PL4 8AA, UK.

(Received: 18 October 2014; accepted: 03 December 2014)

A preliminary study was conduct to determine the effect of iso-osomatic water deficit on three fig (*Ficus carica* L.) cultivars using mannitol as a casual agent for drought stress. The shoots of Fig cultivars (i.e., Black Mission, Brown Turkey and Brunswick) were sub-cultured on MS medium supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} 2iP at different concentrations of mannitol (0.0, 50, 100, 150, 200, 250, and 300 mM L^{-1}) for 35 days under *in vitro* culture conditions. Number of newly formed shoots, shoot length, leaves number per shoot, necrosis %, fresh weight, dry weight, chlorophyll content, relative water content were determined at regular intervals. The Obtained results showed that all growth parameters were reduced by increasing the mannitol concentration except for the percentage of necrosis % which was increased with a significant difference among all treatments. Black Mission was found to resist Mannitol up to concentration of 300 mM L^{-1}, while Brown Turkey and Brunswick cultivars was found to resist mannitol up to the concentration of 250 mM L^{-1}only , where both of this cultivars was not survived under 300 mM L^{-1}. Genomic DNA of the *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick were extracted and were used in performing Randomly Amplified polymorphic DNA (RAPD) and inter simple sequence repeat (*ISSR) markers*. Three arbitrary oligonucleotide primers showed that the number of amplified fragments different from one cultivars to another and this indicating that the fig cultivars are not always identical in their DNA ability to be amplified, which may due to unsimilarity in their genetic structure and common selection history. The use of RAPD molecular markers will be more effectiveness than ISSR to distinguish between the different fig cultivars.

Key words: Fig (*Ficus carica L.*), Drought, Mannitol, Proline, Chlorophyll, Polymorphism, Molecular markers.

Fig (*Ficus carica* L.) is considered as one of the earliest cultivated fruit trees (Bacha *et al.*, 1993). The fig grows well and produces the best quality fruit in drier warm-temperate climates (El-Rayes, 1995). Thus, wild forms of fig are found in Mediterranean countries such as Turkey, Syria, Tunisia, Algeria, Egypt, in Gulf Arabia, Iran, Asia Minor as well as in Central Asia. In Saudi Arabia the fig groves cover most areas in the desert of Saudi Arabia. Different cultivars of fig (*Ficus carica* L.) such as Black Mission, Brown Turkey

* To whom all correspondence should be addressed.
E-mail; egharib@kau.edu.sa

and Brunswick are grown occasionally in specialized orchards of Saudi Arabia such as Gizan, Albaha and Hofuf, and mainly as individual trees in farms.

The cultivated area of fig are usually subjected to degradation and shortages as a result of biotic and abiotic stress and the allowance of the number of fig genotypes selected and maintained , this led to lack of yield production by more than 50% (Zare *et al.,* 2009). Abiotic stress, especially drought stress is one of the critical environmental worldwide problem, seriously constraining global crop production (Pan *et al.,* 2002). Drought stress results in plant water deficits, which is develop as a consequence of water loss from the leaf as the stomata open to allow the uptake of gases from the atmosphere for photosynthesis (Jaleel *et al.,* 2009 ; Fernandez *et al.,* 2006). As a result of this problem, it is imperative to establish breeding programmes aimed to the selection of the fig germplasm for high quantity and quality yield against these stresses environment.

Selection to the best or desirable genotypes is considered an effective plant breeding methods to maintain the production of degradation under environmental stress, but the selection under field conditions suffers from such several disadvantages as extensive labour cost, take a long time, as well the risk of losses due to pathological environmental threats and also affected by environment conditions (Vall *et al.,* 2013). While, data obtained under *in situ* conditions are more accurate than *ex situ* results (Clavel *et al.,* 2005). In the case of programs involving water stress tolerance improvement by *in vitro* selection, Polyethylene glycol (PEG), sucrose, mannitol or sorbitol are the best known selective agents that increase the osmotic pressure in culture media (Manoj *et al.,* 2011)

Artificial induction of drought stress is accomplished using osmotic adjustment chemicals like Mannitol, it is a member of sugar alcohols, that stimulated water deficit by modify the osmotic potential of nutrient solution culture without occasion direct physiological injury (Zang and Komatsu, 2007). It has been reported as an effective osmoticum which only controls the osmotic potential without membrane injury (Ahmad *et al.,* 2007). A series of experiments with mannitol, were performed with excised rice seedling segments and sugarcane shoots grown *in vitro* by Nishimura *et al.,* (2011) and Suriyan and Chlrempol (2008), respectively.

One of the mechanisms used by fruit trees to survive drought is adapt to this stress by reduction in some traits such as growth rate, leaf expansion and stem elongation (Gholami and Rahemi, 2009). Moreover, the accumulation of solutes such as glycine and proline has been linked to water stress, salinity and other abiotic plant stresses (Ashraf and Harris, 2004; Munns and Tester, 2008; Lu *et al.,* 2009), indicating an essential role for these solutes in tolerance to these stresses. Proline accumulates under drought stress and acts as a reserve source of carbon, nitrogen and energy during recovery from stress (Anjum *et al.,* 2011; Watanabe *et al.,* 2000; Chen *et al.,* 2007). Relative water content is considered a measure of plant water status, reflecting the metabolic activity in tissues and used as a most meaningful index for dehydration tolerance (Anjum *et al.,* 2011). With severe stress, the photosynthetic machinery may be damaged, leading to a reduction in the rate of photosynthesis per unit area of leaf and chlorophyll content relying on the period and intensity of drought stress(Kyparissis *et al.,* 1995; Zhang and Kirkham, 1996).

For genotype identification, morphological characters such as leaf, fruit weight, shape and colors were used in phenotypic observations to characterize the genetic diversity of almond species, but a poorness of polymorphism ratio is detecting , since these morphological traits are extremely affected by the abiotic and biotic conditions and/or the plant growth stage (Sorkheh *et al.,* 2007; Zeinalabedinin *et al.,* 2008) . To conquer this nuisance, the employ of molecular markers are widely used in studies of genetic diversity. molecular markers offer numerous advantages over conventional alternatives based on morphological traits be-cause these markers are stable and detectable in all plant tissues, regardless of environmental conditions and developmental stage. The main advantages of molecular markers are the re-duced time required for the genetic study of individuals (Agarwal *et al.,* 2008; Gomes *et al.,* 2010) and the possibility of evaluation during seed or seedling stages. Various molecular markers Simple Sequence Repeats(SSR), randomly

amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), restriction length polymorphism (RFLP), and mitochondrial DNA (RFLP) have been used for DNA fingerprinting studies in fig as well as for germplasm characterization and analysis of genetic diversity in populations (Khadari *et al.*, 2004; Salhi *et al.*, 2004; Achtak *et al.*, 2009; Aradhya *et al.*, 2010; Ikegami *et al.*, 2009; Vall *et al.*, 2013). This study has assured a few advantage since they pliable distinct markers and testament responsible to inwardly manage this important genetic resource. The resulting information will participate to the congregation of background genetic information which may then expedite the selection of a suitable breeding program.

Locally, there is no enough literature or researches regarding the performance of fig under water deficit. Thus, the first goal of this study was to investigate the *in vitro* response of different cultivars of fig (*Fig carica* L.) when begin subjected to drought stress using mannitol as osmotic regulators . A second goal was to determine the effect of this stress on the genetic material of fig cultivars obtained from various location of Saudi Arabia using ISSR and RAPD markers as well as establish a molecular database for fig breeding programs.

MATERIALS AND METHODS

In vitro propagation of fig (*Ficus carica* L.)

Plants were established *in vitro* from shoot tips as reported by (Hemaid *et al.*, 2000; 2010), then propagated by node microcuttings. The explants employed were shoots of the *Ficus carica* L. cultivars (i.e., Black Mission, Brown Turkey and Brunswick) from previous shoot-tip cultures maintained in MS medium supplemented with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, then shoots (about 1-2 cm in length) were subcultured every 4-5 weeks in 250 cm3 jar containing 40 cm of Murashige and Skoog (MS) solid medium supplemented with 3% sucrose , 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} 2iP as described by (**Ehab *et al.*, 2014**). The pH of medium was adjusted to 5.8 using 0.1N NaOH or HCl and the medium was solidified with 0.25% phytagel before autoclaving. The cultures were incubated at 25 ± 1°C in a 16h photoperiod under 50 µmol $m^{-2}s^{-1}$ illumination supplied by cool, white fluorescent light.

Drought tolerance Assessment

In vitro selection procedure under drought stress using mannitol

The shoots of *Ficus carica* L. cultivars (i.e., Black Mission, Brown Turkey and Brunswick) were subcultured on MS medium supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} 2iP (Ehab *et al.*, 2014) with different concentrations of Mannitol (sigma) at 0.0, 100, 150, 200, 250 and 300 mM L^{-1}), respectively to study the effect of osmotic stress on free water deficit as a casual agent for drought stress. Osmotic agents were added to media before pH adjustment then osmotic stress level was measured in all media using electric osmometer. Each treatment consisted of 4 replicates and each experiment was repeated twice. Differences between individual treatments were determined with the least significant difference (LSD) at 0.05 level of probability for all traits under study. After five weeks, the explants were collected and washed for 2 min with distilled water to remove medium dried on filter paper and either used to measurement different growth traits or stored at -20°C for later use.

Data measurement

Relative Water Content (RWC)

For measurement RWC fresh leaves were taken from each cultivars and each replication at after multiplication stage and weighted immediately to record fresh weight (FW). Then they were placed in distilled water for 4 h and then weighted again to record turgid weight (TW), and subjected to oven drying at 70°C for 24 h to record dry weight (DW). The RWC was calculated using the following equation (**Dhopte and Manuel, 2002**): RWC = (FW – DW) / (TW –DW) × 100.

The relative chlorophyll (Chl) content

Was measured with a portable leaf chlorophyll meter (*SPAD 502*, Japan).

Molecular analysis Using RAPD and ISSR marker

Genomic DNA Extraction

DNA was extracted from leaf tissue from each using a Cetyltrimethyl ammonium bromide (CTAB) method according to Maniatis *et al.* (1982). One gram of frozen durum wheat leaves from each entry were ground in cold pestle mortar with 1 ml buffer (100 mM Tris-HCl pH 8.0, 20mM EDTA, 1.4 M NaCl, 0.2% PVP40 (w/v), 0.2% (v/v) 2-

mercaptoethanol) , mixed ,transferred to 500 µl eppendorf tube and incubate at 65 ºC for one hours. After incubation the mixture was centrifuge for 20 minutes with 4000 rpm at room temperature degree. Supernatant was taken and RNAse 1:1000 dilution of RNAse (100 mg ml^{-1}) was added and keep it at 37 ºC at 30 minute, then mixed with the same volume of chlorophorm-isomylalcohol (24:1) and centrifuged at 4000 rpm for 30 minutes. DNA was precipitated by the addition of 2/3 volume of cold isopropanol for overnight in 4ºC. the supernatant was removed from the tube and the pellet was washed with wash buffer (70 % ethanol), centrifuged again for 10 minutes with 1000 rpm at 20ºC and the pellet was dried under vacuum. The DNA pellet was resuspended in 100 µl of deionized H_2O and incubated at 50ºC for 15 min, centrifuged for 5 min with 1000 rpm at 20ºC then the solution was transferred to a new Eppendorf tube. DNA quantity was estimated spectrophotometrically by measuring absorbance 260 nm.

Polymerase Chain Reaction (PCR):

After checking the concentration of genomic DNA by agarose gel electrophoresis for all cultivars of fig (*Ficus carica* L.). Two PCR-based techniques, RAPD and ISSR, were used to detect a marker related to drought tolerance according to Caliskan *et al*., (2012) and Chatti *et al*., (2010), respectively.

DNA amplification

Ten arbitrary 10-base primers of RAPD (Operon Technologies Inc., Alameda, California) and ten primers of ISSR (Fermentas GMBH, Germany) were used for polymerase chain reaction (PCR) (Table 1), following the protocol of Williams *et al*. (1990), with minor modifications. Amplification reactions were performed with 25 µl of 10× assay buffer (Stratagene), 2.0 µl of 1.25 mM each of dNTP's (Pharmacia), 15 ng of the primer, 1× Taq polymerase buffer, 0.5 units of Taq DNA polymerase (Genei, India), 2.5 mM $MgCl_2$, and 30 ng of genomic DNA. DNA amplification was performed in a Perkin Elmer Cetus 480 DNA Thermal Cycler (Perkin Elmer Ce tus, Norwalk, Conn, USA) programmed for 45 cycles as follows: 1st cycle of 3.5 min at 92°C, 1 min at 35°C, 2 min at 72°C; followed by 44 cycles each of 1 min at 92°C, 1 min at 35°C, 2 min at 72°C followed by one final extension cycle of 7 min at 72°C. The amplification products were size separated by electrophoresis in 1.2% (w/v) agarose gels with 0.5× TBE buffer, stained with ethidium bromide, and photographed under UV light.

Amplified DNA Marker Scoring

Amplified DNA markers were scored as present or absent in each cultivar. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored.

Statistical analysis.

Analysis of variance (ANOVA) was executed and treatment means were compared by least significant difference (LSD) test at 5% probability level using MSTATC software according to Freed and Eisensmith (1986).

RESULTS AND DISCUSSION

Growth and physiological markers

Effect of drought, using different concentration of Mannitol, on growth characters;

Table 1. List of RAPD and ISSR primers used for amplification of different DNA obtained from *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick under drought stress.

Sequence (5'———3')	ISSR primers	Sequence (5'———3')	RAPD primers
(GA) 6GG	HB 08	5'- GGCATCGGCT-3'	OPAI-01
(GT) 6GG	HB 09	5'- AGCCGTTCAG-3'	OPAI-02
(GA) 6CC	HB 10	5'-CCCTACTGGT -3'	OPAD-07
(GT) 6CC	HB 11	5'- GGTTCCTCTG-3'	OPAD-13
(CAC) 3GC	HB 12	5'- TTTGCCCCGT-3'	OPAD-15
(GAG) 3GC	HB 13	5'-CTTGGCACGA-3'	OPAD-19
(GTG) 3GC	HB 15	5'-CTGTTGCTAC-3'	Tube O-03
(CT) 8TG	814	5'-CCCAGTCACT-3'	Tube O-05
(CT) 8AC	844A	5'-CCACGGGAAG-3'	Tube O-06
(CT) 8GC	844B	5'-CTCGCTATCC-3'	Tube O-18

shoot length, number of new shoots, leaves no. / shoot, fresh weight, and dry weight after four weeks growth periods on MS medium supplemented with 3.0 mg L^{-1} BA and 0.5 mg L^{-1} 2iP were reported to *Ficus carica* L cultivars Black Mission, Brown Turkey and Brunswick after four weeks of treatments (Fig 1, 2 and 3).

Generally, drought stressed fig plant of Brown Turkey and Brunswick grown under 300 g L-1 were found a 100% death and the data of all parameters, except necrosis %, were undetected , only Black Mission was survived. shoot length, number of new shoots, leaves no. / shoot, fresh weight, and dry weight were decreased with the increased mannitol concentration in the medium except necrosis % (Table 2 and 3). The plantlets length was ranged from 0.20 to 2.78 cm and the greatest plantlets length was found under control treatment with value (2.56, 2.55 and 2.78 cm) of *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick, respectively. The smallest value was recorded by 250 mM L^{-1} mannitol compared with other treatments (Table 2). Whereas, the number of newly formed shoots of *Ficus carica* L cultivars Brown Turkey and Brunswick exhibited a significant decrease under different mannitol concentrations. However, the highest value was recorded by control (0.0) and 50 mM L^{-1}, while the other treatments came in between compared with other treatments (Table 2). Leaves no. per shoot was increased with the increase in mannitol concentration. Maximum leaves no./shoot (7.38, 6.82 and 7.63) was obtained at control treatment of cvs. Black Mission, Brown Turkey and Brunswick, respectively and the minimum fresh weight (0.25) was obtained at 300 g L^{-1} mannitol for Black Mission while at this concentration cultivar Brown Turkey and Brunswick both died (Table 2).

Black Mission, Brown Turkey and Brunswick showed significant genetic variability (Pd"0.05) in necrosis under (05, 100, 150, 200, 250 and 300 mM L^{-1}) mannitol , where no any necrosis was observed under control treatment (Table 2). The rate of necrosis increased sharply after added the mannitol in the MS medium in most of the cultivars, but Brown Turkey and Brunswick had slightly higher rate of necrosis % (100 %) than Black Mission fig cultivars (65.9 %). Similar results were also reported in durum wheat (Farshadfar *et al.*, 2012; Mahmood *et al.*, 2012). El-Houssine and Mohamed (2012) considered callus necrosis percentage as an indicator of tissue culture intolerance to osmotic stress induced by osmotic stress agents such as PEG.

For fresh and dry weight , the results indicated that the effect of mannitol differed significantly , depending on the concentration of mannitol added to medium, but the different between control treatment and 50 mM L^{-1} mannitol was not significant (Table 3). In addition, fresh and dry weights in mild-drought (50-100-150 mM L^{-1} mannitol) were maintained better than those in extreme drought conditions (200-250-300 mM L^{-1} mannitol). The highest value for fresh weight was obtained at control treatment of three cvs., while for dry weight 50 mM L^{-1} recorded highest value (1.28 and 1.30 g) only for Black Mission and Brunswick, respectively. The minimum fresh and dry weight (1.84 and 1.00 g) was obtained at 300 mM L^{-1} mannitol of Black Mission whiles the death

Table 2. Effect of different concentrations of mannitol on growth of *in vitro* micoshoots of *Ficus carica* L cv. Brown Turkey grown on medium supplemented with 3 mg L-1 BAP and 0.5 mg L^{-1} 2iP after five weeks growth periods

Mannitol (mM L^{-1})	Number of newly formed shoots			Shoot length (cm)			Leaves number /shoot			Necrosis %		
	BM	BT	BS	BM	BT	BS	BM	BT	BS	BM	BT	BS
0	7.24a	6.00b	6.37b	2.56a	2.55a	2.78a	7.38a	6.82b	7.63a	0.00i	0.00i	0.00i
50	7.14a	5.64c	5.86c	2.46ab	2.28b	2.48ab	7.14a	6.50b	7.35a	5.20g	7.28g	8.23g
100	6.25b	5.21c	5.22c	1.55bc	1.34c	1.28c	6.65b	5.84c	5.98c	11.2f	16.5f	15.8f
150	4.23d	2.88e	2.55e	1.34c	1.25c	1.00d	5.65c	4.62d	4.54d	52.5d	44.8e	43.6e
200	2.22e	0.23g	1.44f	0.78e	0.65e	0.55e	4.52d	3.55e	3.82e	62.8c	59.5d	58.5d
250	1.08f	0.20g	0.37g	0.58f	0.26f	0.24f	0.54f	0.24f	0.35f	86.8b	78.2b	79.3b
300	0.54g	0.00h	0.00h	0.20g	0.00h	0.00h	0.25f	0.00g	0.00g	65.9c	100a	100a

of plantlets at this concentration of cultivars Brown Turkey and Brunswick (Table 3). Same results , but under salt stress, were obtained by Benmahioul *et al.*, (2009) where they found that pistachio plantlet growth decreased as well as the fresh and dry weights with salinity concentrations.

On the other hand, with respect to physiological traits, this study addressed the effect of Mannitol concentration on chlorophyll and relative water content in fig cultivars. The results indicated that, drought stress caused a significant decrease in chlorophyll and a decline in chlorophyll content was observed in all cultivars due to mannitol concentration increased (Table 3). Maximum chlorophyll content 65.72 µg g^{-1} was obtained at zero level of mannitol in cv. Brunswick While the lowest content value for chlorophyll content was 37.50 µg g^{-1} FW at 300 mM L^{-1} mannitol in Black Mission whiles no data recorded for Brown Turkey and Brunswick because it cannot survived under this concentration. The reduction on the photosynthetic abilities in response to water deficit induced osmotic stresses has been widely investigated in different crops (Cha-um *et al*., 2007a; Wahid and Ghazanfar 2006; Cha-um and Kirdmanee, 2009a; Cha-um *et al*., 2009b). one of the explanation of this decrease in chlorophyll content as affected by water deficit is that drought stress by producing reactive oxygen species (ROS) such as O_2 and H_2O_2, can lead to lipid peroxidation and consequently, chlorophyll destruction (Ganji *et al.*, 2012). Also, by the increasing the reflectance of the incident radiation, due to change the leaf colour from green to yellow, it seem lead to protect the photosynthetic system against stress (Schelmmer *et al.*, 2005)

Sinclair and Ludlow (1985) proposed that RWC was better measure for plant's water status than thermodynamic state variable (water potential, turgor potential and solute potential).Concerning

Fig. 1. Shoot multiplication of *Ficus carica* L. cvs. of Black Mission, Brown Turkey and Brunswick on MS medium supplemented with BA and 2ip with 200 g L^{-1} Mannitol

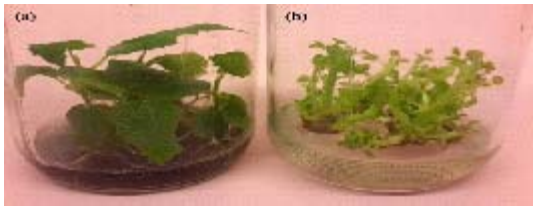

Fig. 2. *Ficus carica* cv. Black Mission (a): MS medium supplemented with 3 mg L^{-1}BAP and 0.5 mg L^{-1} 2ip (b): MS medium supplemented with 1mg L^{-1} IBA, 0.5 mg L^{-1} NAA and 2 g L^{-1} activated charcoal (Healthy plantlets with 150 mM L^{-1} mannitol).

Table 3. Effect of different concentrations of mannitol on fresh weight, dry weight, chlorophyll contents and relative water contents of in vitro micoshoots of Ficus carica L cv. Brown Turkey grown on medium supplemented with 3 mg L-1 BAP and 0.5 mg L-1 2iP after five weeks growth periods

Mannitol (mM L^{-1})	Fresh weight / five explants (g)			Dry weight/five explant (g)			Chl content [SPAD]			Relative Water Content (%)		
	BM	BT	BS	BM	BT	BS	BM	BT	BS	BM	BT	BS
0	7.24a	6.00b	6.37b	2.56a	2.55a	2.78a	7.38a	6.82b	7.63a	0.00i	0.00i	0.00i
50	7.14a	5.64c	5.86c	2.46ab	2.28b	2.48ab	7.14a	6.50b	7.35a	5.20g	7.28g	8.23g
100	6.25b	5.21c	5.22c	1.55bc	1.34c	1.28c	6.65b	5.84c	5.98c	11.2f	16.5f	15.8f
150	4.23d	2.88e	2.55e	1.34c	1.25c	1.00d	5.65c	4.62d	4.54d	52.5d	44.8e	43.6e
200	2.22e	0.23g	1.44f	0.78e	0.65e	0.55e	4.52d	3.55e	3.82e	62.8c	59.5d	58.5d
250	1.08f	0.20g	0.37g	0.58f	0.26f	0.24f	0.54f	0.24f	0.35f	86.8b	78.2b	79.3b
300	0.54g	0.00h	0.00h	0.20g	0.00h	0.00h	0.25f	0.00g	0.00g	65.9c	100a	100a

BM= Black Mission, BT= Brown Turkey and BS= Brunswick
Averages followed by the same letter within a column are not significantly different at P<0.05

the effect of mannitol concentration in the regeneration medium on another physiological traits such as RWC, data in (Table 3) showed that, by add 250 mM mannitol per litter of MS medium, the RWC was reduced from 81.67, 86.56, 87.46 (control) to 69.85, 69.75, 68.82 in Black Mission, Brown Turkey and Brunswick, respectively. This reduction in RWC may be due to at the cellular level, plants attempts to alleviate the damaging effects of stress by altering their metabolism to cope with stress (Tarek *et al.,* 2008). Statistically analysis showed that, non significant differences were found between the genotypes under investigation but clear differences was found between concentration of mannitol and it is relation with RWC. Similar results were demonstrated where the relative water content in the callus tissues was positively significantly decreased with 0, 100 , 200 and 300 mM mannitol contained in MS medium (Errabi *et al*., 2006; Errabi *et al*., 2007; Tarek *et al.*, 2008). Therefore these genotypes which maintained higher RWC under stress conditions is believed to be more droughts tolerant and gave higher yielding than others. These results recognized RWC as a beneficial drought tolerance indicator and may be used as selection criteria in breeding program. There are many reports which show physiological and morphological changes in response to drought stress, are used as potential and rapid tool for screening for drought tolerance (Nable *et al*., 1999; Robertson *et al*., 1999; de Silva and de Costa, 2004; Inman-Bamber and Smith, 2005; Smit and Singels, 2006; Silva *et al*., 2007), especially under *In vitro* environment.

Fig. 3. Rooting of *Ficus carica* cvs. Black Mission, Brown Turkey and Brunswick on MS medium supplemented with 1mg L^{-1} IBA and 0.5 mg L^{-1} NAA (Healthy plantlets with 50 g L^{-1} mannitol).

Molecular analysis using RAPD and ISSR marker

Genomic DNA of the *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick were extracted and were used in performing Randomly Amplified polymorphic DNA (RAPD) and inter simple sequence repeat (*ISSR) markers*. three arbitrary oligonucleotide primers, number of fragment amplified using these different primers showed that the number of amplified fragments different from one cultivars to another indicating that all cultivars are not always identical in their DNA ability to be amplified and these primers have

Table 4. Polymorphism rate for *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick using OPAI-01, OPAD-15 and Tube O-06 RAPD primers.

Primer code	Sequence (5'————————- 3')	Number of bands	Number of Polymorphic markers	Polymorphism (%)
OPAI-01	5'- GGCATCGGCT-3'	87	21	24.1
OPAD-15	5'- TTTGCCCCGT-3'	92	27	29.3
Tube O-06	5'-CCACGGGAAG-3'	80	18	22.5
	Total	259	66	

Table 5. Polymorphism rate for *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick using HB 11, HB 15 and 844A ISSR primers

Primer code	Sequence (5'————————- 3')	Number of bands	Number of Polymorphic markers	Polymorphism (%)
HB 11	(GT) 6CC	78	5	6.4
HB 15	(GTG) 3GC	64	31	48.4
844A	(CT) 8AC	66	11	16.7
	Total	208	48	

amplified 259 PCR bands with RAPD marker 208 PCR bands with ISSR marker and (Table 4 and 5).

The results of RAPD and ISSR analysis using primers (OPAI-01, OPAD-15,Tube O-06) and (HB11, HB15, 844A), respectively are illustrated in figure (4 and 5). The OPAAD-15 and HB15 primer recorded the highest percentage polymorphism (29.3% ; 48%) as it revealed 27 and 31 polymorphic

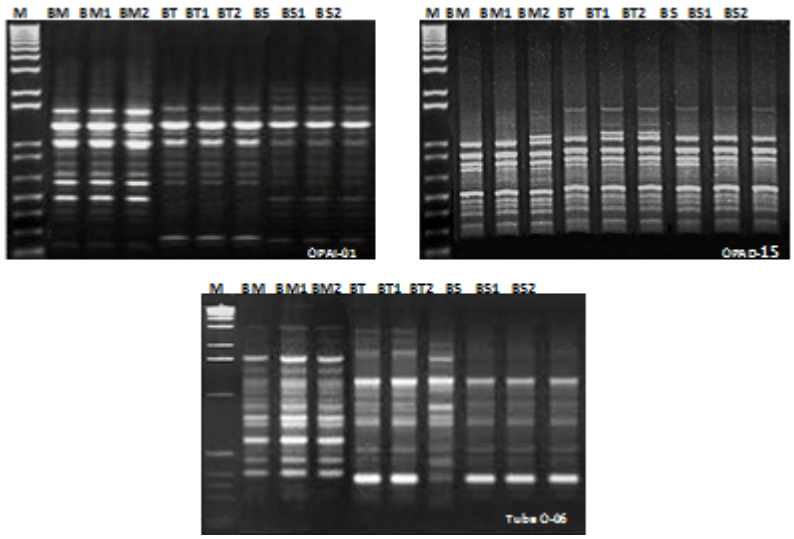

Fig. 4. DNA banding pattern generated by RAPD-PCR with different primers OPAI-01, OPAD-15 and Tube O-06 in *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick. Lane M: 1kb plus DNA ladder; (BM) Black Mission control, (BM1) Black Mission with 200 mM /L manitol, (BM2) Black Mission with 250 mM /L manitol, (BT) Brown Turkey control, (BT1) Brown Turkey with 200 mM /L manitol, (BT2) Brown Turkey with 250 mM /L manitol, (BS) Brunswick control, (BS1) Brunswick with 200 mM /L manitol and (BS2) Brunswick with 250 mM /L manitol.

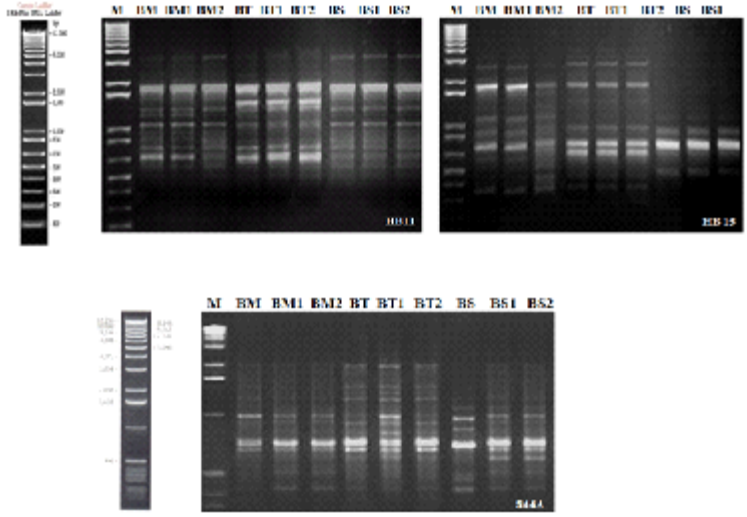

Fig. 5. DNA banding pattern generated by ISSR-PCR with different primers HB11 , HB15 and 844A in *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick. Lane M: DNA marker (1kb plus DNA ladder); (BM) Black Mission control, (BM1) Black Mission with 200 mM /L manitol, (BM2) Black Mission with 250 mM /L manitol, (BT) Brown Turkey control, (BT1) Brown Turkey with 200 mM /L manitol, (BT2) Brown Turkey with 250 mM /L manitol, (BS) Brunswick control, (BS1) Brunswick with 200 mM /L manitol and (BS2) Brunswick with 250 mM /L manitol.

bands in 92 and 64 amplified fragments, respectively. The Tube O-06 primer amplified 80 bands in *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick. In RAPD analysis the Tube O-06 primer recorded the lowest percentage polymorphism (22.5%) as it revealed 18 polymorphic bands in 80 amplified fragments, while in ISSR analysis the lowest level of polymorphism (6.4 %) was recorded for HB11 primer. These results agree with Rashed *et al*., 2010., they reported that six primers only gave a polymorphism with wheat genotypes, which four primers out of them developed molecular markers of drought tolerant wheat. The five primers produced multiple band profiles with a number of amplified DNA fragment ranging from Zero to eleven. Three primers P18, P29 and P39 were reacted and generated PCR product with all genotypes, whereas primer P24 and P86 reacted only with twelve and fourteen genotypes, respectively.

CONCLUSION

In the present study, all traits studies showed a progressive decrease consistently with increase in mannitol concentration in the culture medium and , conversely, raise the percentage of necrosis %., Drought-stressed fig plants of Brown Turkey and Brunswick grown under 300 mM L^{-1} mannitol were found a 100% death. In conclusion, Black Mission cultivar was very tolerance , than Brown Turkey and Brunswick, to water deficit as it had a maximum value for most of the traits under different treatments of mannitol. It is evident from this study that the RAPD and ISSR assay is important since it is relatively easy to obtain valuable data and it can be useful in fig breeding programmes, where breeders can select related or unrelated parental germplasm to maximize variability in fig breeding programme under abiotic stress.

ACKNOWLEDGMENTS

This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant number (3-965-35-RG). The authors, therefore, acknowledge with thanks DSR technical and financial support

REFERENCES

1. Achtak H, Oukabli A, Ater M, Santoni S. Microsatellite markers as reliable tools for fig cultivar identification. *J. Am. Soc. Hort. Sci.* 2009; **134**: 624-631.
2. Agarwal M, Shrivastava N and Padh H. Advances in molecular marker techniques and their applications in plant sciences. *Plant Cell Rep*.2008; **27**: 617-631.
3. Ahmad M.S.A., Javed F., Ashraf M., Iso-osmotic effect of NaCl and PEG on growth, cations and free proline accumulation in callus tissues of two indica rice (*Oryza sativa* L.) genotypes. *Plant Growth Regul*, 2007; **53**, 53-63.
4. Anjum, S.A., Xie, X., Wang, L.C., Saleem, M.F., Man, C. and Lei, W. Morphological, physiological and biochemical responses of plants to drought stress. *African Journal of Agricultural Research*. 2011; **6**(9): 2026–2032.
5. Aradhya M, Stover E, Velasco D, Koehmstedt A. Genetic structure and differentiation in cultivated fig (*Ficus carica* L.). *Genetica*,2010; **138**: 681-694.
6. Ashraf M, Harris P. Potential biochemical indicators of salinity tolerance in plants. *Plant Science*,2004; **166**, 3-16.
7. Bacha M, Shaheen M, Farahat F. Studies on the Adaptability of Some Introduced Fig Cultivars to Riyadh Region, Saudi Arabia. .*IKAU: st«, EliI' .. Arid lund Agric, Sci.,* 1993; **4**, pp. H7-94 (141.~ A.H. I 1993 A.D .).
8. Benmahioul B, Daguin F, Kaid-Harche M. Effects of salt stress on germination and *in vitro* growth of pistachio (*Pistacia vera* L.). *Comptes Rendus Biologies*, 2009; **332**(8):752-758.
9. Caliskan O., Polat A. A., Celikkol P. and Bakir M. Molecular characterization of autochthonous Turkish fig accessions. *Spanish Journal of Agricultural Research*, 2012; **10**(1): 130-140.
10. Chatti K, Baraket G, Ben A, Saddoud O. Development of molecular tools for characterization and genetic diversity analysis in Tunisian fig (*Ficus carica*) cultivars. *Biochem. Genet.* 2010; **48**: 789-806.
11. Cha-um, S. and C. Kirdmanee. Proline accoumulation, photosynthetic abilities and growth characters of sugarcane (*Saccharum offinarum* L.) plantlets in response to iso-osmotic salt and water-deficit stress. *Agric. Sci. China*, 2009a; **8**: 51-58.
12. Cha-um, S. and C. Kirdmanee. Effect of salt stress on proline accumulation, photosynthetic

ability and growth characters in two maize cultivars. *Pak. J. Bot.*, 2009b; **41**: 87-98.

13. Cha-um, S., K. Supaibulwatana and C. Kirdmanee. Glycinebetaine accumulation, physiological characterizations, and growth efficiency in salt tolerant and salt sensitive lines of indica rice (*Oryza sativa* L. spp. *indica*) response to salt stress. *J. Agron. Crop Sci*., 2007a; **193**: 157-166.
14. Chen Z, Cuin TA, Zhou M, Twomey A, Naidu B P, Shabala S. Compatible solute accumulation and stress mitigating effects in barley genotypes contrasting in their salt tolerance. *Journal of Experimental Botany*, 2007; **58**; 4245-4255.
15. Clavel, D., Drame, N.K., Roy-Macauley, H., Braconnier, S., Laffray, D., Analysis of early responses to drought associated with field drought adaptation in four Sahelian groundnut (Arachis hypogaea L.) cultivars. *Environ. Exp. Bot.* 2005; **54**, 219–230.
16. de Silva, A.L.C. and W.A.J.M. de Costa. Varietal variation in growth, physiology and yield of sugarcane under two contrasting water regimes. *Trop. Agric. Res.*, 2004; **16**: 1-12.
17. Dhopte AM, Manuel LM. Principles and Techniques for Plant Scientists. 1st Edn., Updesh Purohit for Agribios (India), Odhpur, IBSN: 81-7754-116-1, pp: 373; 2002.
18. Ehab, MR., Soliman H., Al-zaharzni H., Howladar S., Fuller, M. Influence of different concentrations of salt stress on *in vitro* multiplication of some fig (*Ficus carcia L.*) cultivars. *Life Science Journal*, 2014; **11**: 386-397.
19. El Houssine Bouiamrine and Mohammed Diouri. Response of durum wheat (*Triticum durum* Desf.) callus culture to osmosis induced drought stress caused by polyethylene glycol (PEG). *Annals of Biological Research*, 2012, (9):4555-4563.
20. El-Rayes R.1995. The fig tree in the Mediterranean region and in Syria. *Options Mediterr*, 2012; **13**: 79-83.
21. Errabii, T., C.B. Gandonou, H. Essalmani, J. Abrini, M. Idaomar and N.S. Senhaji. Effect of NaCl and mannitol induced stress on sugarcane (*Saccharum* sp.) callus cultures. *Acta Physiol. Plant.*, 2007; **29**: 95-102.
22. Errabii, T., C.B. Gandonou, H. Essalmani, J. Abrini, M. Idomar and N.S. Senhaji. Growth, proline and ion accumulation in sugarcane callus cultures under drought-induced osmotic stress and its subsequent relief. *Afri. J. Biotechnol.*, 2006; **5**: 1148-1493.
23. Farshadfar Ezatollah, Bita Jamshidi1, Kianosh Cheghamirza, Jaime A. Teixeira da Silva. Evaluation of drought tolerance in bread wheat(*Triticum aestivum* L.) using *in vivo* and *in vitro* techniques. *Annals of Biological Research*, 2012, **3**(1):465-476
24. Fernandez, J.A., Balenzategui, L., Ba˜nón, S., Franco, J.A., Induction of drought tolerance by paclobutrazol and irrigation deficit in Phillyrea angustifolia during the nursery period. *Sci. Hortic.* 2006; **107**, 277–283.
25. Freed, R.D. and S.P. Eisensmith. MSTAT microcomputer Statistical Program. Michigan State University of Agri. Michigan, Lansing, 1986; USA.
26. Ganji F. Arjenaki1, R. Jabbari1, A. Morshedi. Evaluation of Drought Stress on Relative Water Content, Chlorophyll Content and Mineral Elements of Wheat (Triticum aestivum L.) Varieties. *Intl J Agri Crop Sci.* 2012; **4** (11), 726-729,
27. Gholami, M., Rahemi, M., Effect of irrigation regimes on water status and photosynthetic parameters of peach-almond hybrid (GF677) seedlings and cuttings. Hortic. *Environ. Biotechnol.* 2009; **50**, 94–99.
28. Gomes Filho A, Oliveira JG, Viana AP, Siqueira APO, et al. Marcadores moleculares RAPD e descritores morfológicos na avaliação da diversidade genética de goiabeiras (*Psidium guajava* L.). *Acta Sci. Agron.* 2010; **32**: 627-633.
29. Hemaid S. Tissue culture technique for production of virus-free fig and its *in vitro* preservation. M.Sc. Thesis, Faculty of Agric Cairo Univ 2000; 145.
30. Hemaid S, Mahdia G, Naglaa A. Efficient transformation and regeneration of fig (*Ficus carica L.*) via somatic embryogenesis . *GM Crops and Food*, 2010; **1**: 42 – 53.
31. Ikegami H, Nogata H, Hirashima K, Awamura M. Analysis of genetic diversity among European and Asian fig varieties (*Ficus carica* L.) using ISSR, RAPD, and SSR markers. *Genetic Res Crop Evol*, 2009; **56**: 201-209.
32. Inman-Bamber, N.G. and D.M. Smith. Water relations in sugarcane and response to water deficits. *Field Crops Res.*, 2005; **92**: 185-202.
33. Jaleel CA, Manivannan P, Wahid A, Farooq M, Al-Juburi HJ, Somasundaram R,Panneerselvam R, Drought Stress in Plants: A Review on MorphologicalCharacteristics and Pigments Composition. *International Journal of Agricultureand Biology*, 2009; **11**: 100-105
34. Khadari B, Oukabli A, Ater M, Mamouni A. Molecular characterization of Moroccan fig germplasm using intersimple sequence repeat and simple sequence repeat markers to establish

a reference collection. *Hort Science,* 2004; **40**: 29-32.

35. Kyparissis, A., Petropoulun, Y. and Manetas, Y. Summer survival of leaves in a soft-leaved shrub (Phlomis fruticosa L., Labiatae) under Mediterranean field conditions: avoidance of photoinhibitory damage through decreased chlorophyll contents. *Journal of Experimental Botany.*1995; **46**: 1825-1831.
36. Lu SY, Chen CH, Wang ZC , Guo ZF, Li HH. Physiological responses of somaclonal variants of triploid bermudagrass (*Cynodon transvaalensis x Cynodon dactylon*) to drought stress. *Plant Cell Reports,* 2009; **28**, 517-526.
37. Mahmood Imran, Abdul Razzaq, Ishfaq Ahmad Hafiz, Shuaib Kaleem, Ahmad Ali Khan, Abdul Qayyum and Muhammad Ahmad. Interaction of callus selection media and stress duration for *in vitro* selection of drought tolerant callus of wheat. *African Journal of Biotechnology,* 2012; **11**(17), 4000-4006
38. Maniatis, T., Fritch, E., Sambroak, J. Molecular Cloning: A laboratory manual. Cold Springer Harbor Laboratory, Cold Springer Harbor, New York 1982.
39. Manoj K, Rajwant K, Rohtas S, Manu P, Dhawan A. Developing stress tolerance plants through in vitro selection-An overview of the recent progress. *Environmental and Experiment Botany* **7**(1):89-98.
40. Munns R, Tester M. Mechanisms of salt tolerance. *Annual Review Plant Biology*, 2008; **59**, 651-681.
41. Nable, R.O., M.J. Robertson and S. Berthelsen. Response of shoot growth and transpiration to soil drying in sugarcane. *Plant Soil*, 1999; **207**: 59-65.
42. Nishimura T., Cha-um S., Takagaki M., Ohyama K., Kirdmanee. Survival percentage, photosynthetic abilities and growth characters of two indica rice (*Oryza sativa* L. spp. *indica*) cultivars in response to iso-osmotic stress. *Spanish Journal of Agricultural Research,* 2011; **9**(1), 262-270
43. Pan, X.Y., Wang, Y.F., Wang, G.X., Cao, Q.D. and Wang, J., Relationship between growth redundancy and size inequality in spring wheat population mulched with clear plastic film. *Acta Phytoecol. Sinica,* 2002; **26**: 177-184.
44. Rashed M. A., Sabry S. R. S., Atta A. H. and Mostafa A. M. Development of RAPD marker associated with drought tolerance in bread wheat (*Triticum aestivum*) *Egypt. J. Genet. Cytol.,* 2010; **39**: 131-142.
45. Robertson, M.J., N.G. Inman-Bamber, R.C. Muchow and A.W. Wood. Physiology and productivity of sugarcane with early and mid-season water deficit. *Field Crops Res.*,1999; **64**: 211-227.
46. Salhi-Hannachi, A., Trifi, M., Zehdi, S. et al. Inter simple sequence repeat finger printings to assess genetic diversity in Tunisian fig (Ficus carica L.). *Genet. Resour. Crop Evol.* 2004a; **51**: 269-275.
47. Schlemmer MR, francis DD, Shanahan JF, Schepers JS. Remotely measuring chlorophyll content in corn leaves with differing nitrogen levels and relative water content. *Agron. J.*, 2005; **97**: 106-112.
48. Silva, M.A., J.L. Jifon, J.A.G. da Silva and V. Sharma. Use of physiological parameters as fast tools to screen for drought tolerance in sugarcane. *Braz. J. Plant Physiol.*, 2007; **19**: 193-201.
49. Sinclair T, Ludlow M, Who taught plants thermodynamics? The unfulfilled potential of plant water potential. *Aust. J. Plant Physiol.* 1985; **12**: 213-217.
50. Smit, M.A. and A. Singels. The response of sugarcane canopy development to water stress. *Field Crops Res.*, 2006; **98**: 91-97.
51. Sorkheh, K. B. Shiran, T. M. Gradziel, B. K. Epperson, P. Martínez-Gómez and E. Asadi, "Amplified Fragment Length Polymorphism as a Tool for Molecular Charac- terization of Almond Germplasm: Genetic Diversity among Cultivated Genotypes and Related Wild Species of Almond, and Its Relationships with Agronomic Traits," *Euphytica*, **156**(3); 327-344.
52. Suriyan, C., Chalermpol, K. Effect of osmotic stress on proline accumulation, photosynthetic abilities and growth of sugarcane plantlets (*Saccharum officinarum* L.). *Pak. J. Bot.*, 2008; **40**: 2541-2552, 2008.
53. Tarek Y. Bayoumi, Manal H. Eid and E. M. Metwali. Application of physiological and biochemical indices as a screening technique for drought tolerance in wheat genotypes. *African Journal of Biotechnology,* 2008; **7** (14), 2341-2352
54. Vall A, Souza1C, Ferreira E, Salgado S, Pasqual M, Cançado1G. Evaluation of genetic diversity in fig accessions by using microsatellite markers. *Genet Mol Res*, 2013; **12**: 1383-1391.
55. Wahid, A. Analysis of toxic and osmotic effects of sodium chloride on leaf growth and economic yield of sugarcane. *Bot. Bull. Acad. Sin.*, 2004; 45: 133-141.
56. Watanabe A, Kojima K, Ide Y, Sasaki S. 2000. Effects of saline and osmotic stress on proline and sugar accumulation in *Populus euphratica* in vitro. *Plant Cell Tissue & Organ Culture,* 63, 199-206.

57. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalsk, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18**: 6531–6535.
58. ZANG X., KOMATSU S., A proteomics approach for identifying osmotic-stress-related proteins in rice. *Phytochem*, 2007; **68**, 426-437.
59. Zare, H., Zare, E., Pir Moradiyan, N., Joukar, L., Mobayen, K.h., Nazari, A., Golkar, G.h. and Kamkar Haghighi, A.A. Reducing damages caused by drought on the rainfed fig cultivation and its economic-social impacts. Seed and Plant Improvement Institute, Karaj, p 40.
60. Zeinalabedini M., K. Majourhat, V. Grigorian, M. Torchi, F. Dicenta, P. Martínez-Gómez, "Comparison of the Use of Morphological, Protein and DNA Markers in the Ge- netic Characterization of Iranian Wild Prunus Species," *Scientia Horticulturae*, 2008; **116**(1), 80-88.
61. Zhang, C-S., Lu, Q. and Verma, D. P. S. Characterization of Ä1-pyrroline-5-carboxylate synthetase gene promoter in transgenic Arabidopsis thaliana subjected to water stress. *Plant Science.* 1997; **129**: 81–89.
62. Zhang, J. and Kirkham, M.B. Antioxidant response to drought in sunflower and sorghum seedlings. *New Phytologist.* 1996; **132**: 361-373.