Appraisal of *in vitro* drought stress among three different cultivars of fig (*Ficus carica* L.) using RAPD and ISSR markers

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Abstract

Response of three cultivars of fig (*Ficus carica* L.) to *in vitro* selection of drought tolerance was the main objective of this study. The effect of water stress induced by mannitol on growth water content, necrosis of *in vitro* cultures and regeneration was investigated on selected fig cultivars. The shoots of fig plants 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) under *in-vitro* culture conditions. The results showed that increasing mannitol concentration in the medium causes a gradual decrease in all growth parameters and plant regeneration efficiency. The results indicated that mannitol can be used as water stress creating agent under *in vitro* conditions and Black Mission cultivar was relatively tolerant to drought stress as compared to Brown Turkey and Brunswick. RAPD and ISSR-PCR with three primers used to distinguish plantlets with regenerated from the mannitol- tolerant and control plantlets. The analysis revealed three primers associated with drought tolerance which can be utilized in breeding programme via marker assisted selection and developing drought tolerant cultivars by genetic transformation.

Keywords: Drought, Fig (*Ficus carica L.*), genetic markers, plantlets, regeneration, similarity matrix.
Abbreviations: 2iP_2-isopentenyl adenine; BAP_benzylaminopurine; Chl _Chlorophyll; DW_dry weight; FW_fresh weight; Kn_ kinetin; IBA_ indole butyric Acid; ISSR_ inter-simple sequence repeat; MS_Murashige and Skoog; NAA_ naphthaleneacetic acid; PCR_polymerase chain reaction; PGRs_ plant growth regulators; RAPD_ randomly amplified polymorphic DNA; RWC_ relative water content; TW_ turgid weight.

Introduction

Fig (*Ficus carica* L.) is considered to be one of the earliest cultivated fruit trees (Bacha et al., 1993) and grows well and produces the best quality fruit in drier warm-temperate climatesn with wild forms of fig found extensively in Mediterranean countries such as Turkey, Syria, Tunisia, Algeria, Egypt, as well as in the Arabian Gulf and in Central Asia (El-Rayes, 1995; Muhammod, 2012). The cultivated areas of fig are usually subjected to biotic and abiotic stress leading to a decrease in yield production sometimes by more than 50% (Zare et al., 2009). Abiotic stress, especially drought stress is one of the critical environmental worldwide problems, seriously constraining global crop production (Pan et al., 2002; Manoj et al., 2011) and is a major limitation to fig yield. Drought stress results in plant water deficits which 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). It would be highly desirable to establish breeding programmes aimed at the selection of fig germplasm for high yield of quality produce under drought stress environments.

Selection of the best or desirable genotypes is considered an effective plant breeding method to maintain production under environmental stress, but the selection under field conditions suffers from such several disadvantages such as extensive labour cost and extended durations as well a risk of losses due to pathological losses and adverse environmental conditions (Vall et al., 2013). While, data obtained under *in situ* conditions are more accurate than *ex situ*, *in vitro* culture techniques can minimize environmental variation during selection due to defined nutrient media, controlled conditions and the homogeneity of stress application (Clavel et al., 2005; Anbar, 2010) and has role to play in breeding programs of perennial fruit trees. Plant breeders have always looked for appropriate and repeatable indicators to screen for drought tolerance (Hasheminasab et al., 2014). 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 and mimic drought stress without causing too many other physiological artefacts (Manoj et al., 2011).

Mannitol is an osmotically active sugar alcohol that is not metabolized by plant cells and be used to simulate water deficit by modification of the osmotic potential of culture media without direct physiological injury from the mannitol itself (Zang and Komatsu, 2007). It has been reported as an effective osmoticum which controls the osmotic potential without membrane injury and is greatly used in germplasm screening of water stress during *in-vitro* regeneration phases (Ahmad et al., 2007; Anbar, 2010; Hannes et al., 2014). Successful experiments with mannitol have been performed with a variety of explants such as seedlings (Nishimura et al., 2011), cotyledons (Abdel-Raheem et al., 2007; Zhaleh et al., 2013), immature embryos (Matheka et al., 2008), shoot tips (Suriyan and Chlrempol, 2008) and callus (Zahidul et al., 2013). Recently studies have concluded that water stress eventually results in the modification of various morphological, physiological and biochemical traits in order to maintain cellular homeostasis during stress conditions (Priyank et al., 2015). One of the mechanisms used by fruit trees to survive drought is to adapt to the stress by a reduction in some growth traits such as growth rate, leaf expansion and stem elongation (Gholami and Rahemi, 2009). Furthermore, relative water content is considered a measure of plant water status, reflecting the metabolic activity in tissues and is can be used as one of the most meaningful indices for dehydration tolerance studies (Anjum et al., 2011) and has been used for assessing drought tolerance (Xing et al., 2004). With severe drought stress, the photosynthetic machinery may be damaged, leading to a reduction in the rate of photosynthesis per unit area of leaf and reduction in chlorophyll content depending on the period and intensity of drought stress (Kyparissis et al., 1995; Zhang and Kirkham, 1996; Neha et al., 2015).

For genotype identification, morphological characters such as leaf morphology, fruit weight, shape and colors have been used in phenotypic observations to characterize the genetic diversity of several species including almond, but a poorness of polymorphism ratio is detected since these morphological traits are highly affected by abiotic and biotic conditions and/or the plant growth stage (Sorkheh et al., 2007; Zeinalabedinin et al., 2008). To ovrcome this dilemma, molecular markers have become widely used in studies of genetic diversity. Molecular markers offer numerous advantages over conventional morphological traits because they are stable and detectable in all plant tissues, regardless of environmental conditions and independent of developmental stage. Furthermore reduced time is required for the genetic study of individuals with molecular markers and there is the possibility of evaluation during seed or seedling stages of the plant (Agarwal et al., 2008; Gomes et al., 2010; Emmanual et al., 2012). Various molecular markers such as 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 natural and synthetic populations (Khadari et al., 2004; Salhi et al., 2004a; Achtak et al., 2009; Ikegami et al., 2009; Aradhya et al., 2010; Vall et al., 2013). These studies have conferred several advantages since they show that the markers are distinct and reliable and are of great assistance to manage important genetic resources. The resulting information of studies of this sort participate in the congregation of background genetic information which may then expedite selection within a suitable breeding program.

There is a dearth of literature or published research regarding the performance of fig under water deficit. Thus, the first goal of the current study was to investigate the *in-vitro* response of different cultivars of fig when subjected to drought stress using mannitol as the osmotic regulator. The second goal was to determine the amount of genetic diversity among selected commonly used genotypes and to determine the genetic relationships between fig cultivars obtained from various locations using ISSR and RAPD markers in order to begin to establish a molecular database for fig breeding programs.

Results and Discussion

Growth and physiological responses

Effect of drought, using different concentration of mannitol, on growth characters; shoot length, number of new shoots, number of leaves, 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 fig cultivars Black Mission, Brown Turkey and Brunswick after four weeks of treatments (Fig 1, 2 and 3).

Generally, mannitol induced drought stress decreased plant growth and vigour of all fig cultivars incrementally as the concentration of mannitol was increased. A concentration of 300 mM was lethal for Brown Turkey and Brunswick cultivars but Black Mission survived this concentration (Tables 2 & 3). The greatest plantlet length was found under the control treatment with values of 2.56, 2.55 and 2.78 cm for cvs. Black Mission, Brown Turkey and Brunswick, respectively. Mannitol decreased shoot length at all concentrations with the smallest value recorded at 250 mM (Table 2) and decreased the number of newly formed shoots. Increasing mannitol concentrations also progressively reduced fresh weight and dry weight (Table 2). Similar 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 increasing salinity concentrations.

Black Mission, Brown Turkey and Brunswick showed significant (p ≤ 0.05) genetic variability in necrosis at all levels of mannitol (Table 2). The rate of necrosis increased sharply after mannitol was added to the MS medium in most of the cultivars, but Brown Turkey and Brunswick had slightly higher rates of necrosis (100 %) compared to Black Mission (65.9 %). Similar genetic variability was also reported in related experiments on 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 polyethylene glycol PEG or mannitol.

With respect to physiological traits, this study addressed the effect of mannitol concentration on chlorophyll and relative water content in fig cultivars. The results showed that drought stress caused a significant decrease in chlorophyll and a decline in chlorophyll content was observed in all cultivars with increased mannitol concentration (Table 3). Maximum chlorophyll content 65.72 μg g^{-1} FW was obtained in cv. Brunswick control cultures whilst the lowest content value for chlorophyll content was 37.50 μg g^{-1} FW at 300 mM mannitol in Black Mission. The reduction of the photosynthetic ability in response to water deficit induced osmotic stresses has been widely investigated in different crops (Cha-um et al., 2007; Wahid and Ghazanfar, 2006; Cha-um and Kirdmanee, 2009a; Cha-um and Kirdmanee

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

RAPD primers	Sequence (5'-------3')	ISSR primers	Sequence (5'-------3')
OPAI-01	5'- GGCATCGGCT-3'	HB 08	5'- GAGAGAGAGAGAGAGG-3'
OPAI-02	5'- AGCCGTTCAG-3'	HB 09	5'- GTGTGTGTGTGTGTGG-3'
OPAD-07	5'-CCCTACTGGT -3'	HB 10	5'- GAGAGAGAGAGACC-3'
OPAD-13	5'- GGTTCCTCTG-3'	HB 11	5'- GTGTGTGTGTGTCC-3'
OPAD-15	5'- TTTGCCCCGT-3'	HB 12	5'- CACCACCACGC-3'
OPAD-19	5'-CTTGGCACGA-3'	HB 13	5'- GAGGAGGAGGC-3'
Tube O-03	5'-CTGTTGCTAC-3'	HB 15	5'- GTGGTGGTGGC-3'
Tube O-05	5'-CCCAGTCACT-3'	814	5'- CTCTCTCTCTCTCTCTTG-3'
Tube O-06	5'-CCACGGGAAG-3'	844A	5'- CTCTCTCTCTCTCTCTAC-3'
Tube O-18	5'-CTCGCTATCC-3'	844B	5'- CTCTCTCTCTCTCTCTGC-3'

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 mM mannitol.

2009b) and one of the explanations of this decrease in chlorophyll content is that drought stress produces reactive oxygen species (ROS) such as O^- and O_2 and H_2O_2 which can lead to lipid peroxidation and consequently, chlorophyll destruction (Ganji et al., 2012). Also, the increased reflectance of incident radiation, due to a leaf colour change from green to yellow can lead to some protection of the photosynthetic system against stress (Schelmmer et al., 2005) Sinclair and Ludlow (1985) proposed that RWC was a better measure for plant water status than thermodynamic state variables such as water potential, turgor potential and solute potential and this technique was used here. Data showed that by adding 250 mM mannitol to 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, to the plants attempts to alleviate the damaging effects of stress by altering their metabolism to cope with stress (Tarek et al., 2008). Statistical analysis showed that overall differences between the genotypes were not significant but clear differences were found between the different concentration of mannitol. Similar results were demonstrated where the relative water content in the callus tissues was significantly decreased with increasing mannitol contained in the MS medium (Errabi et al., 2006; Errabi et al., 2007; Tarek et al., 2008). Genotypes which maintain higher RWC under stress conditions are believed to be more droughts tolerant and are potentially 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, and several have been suggested as potential and rapid tools 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* environmental conditions.

Chemical analysis

Mannitol-stressed fig showed a gradual reduction in the concentrations of all tested minerals (i.e., K, P, Ca, Mg, Na, Fe, Zn, Mn and Cu) as the concentration of mannitol in the growth medium increased (Table 4). This was the same with all tested cultivars (Black Mission, Brown Turkey and Brunswick). Black Mission had the highest concentrations of minerals under each mannitol concentration and was statistically significant to the other two cultivars suggesting that this was the most tolerant cultivar. This result agrees with Osuagww and Edeoga (2012) who indicated that the concentration of mineral elements in plants may be influenced by environmental factor such as water stress. Parvaneh and Seyed (2012) also indicated that with increasing drought level meaningful increases of Na and meaningful decreases of K, Ca, Mg, Mn and Fe content was observed. Water stress develops when the water efflux from the plant is greater than the water influx into the plant and, as found in our study, this can be controlled caused by mannitol. Water deficit are very common in the production cycle of most crops, and numerous studies have indicated that it can have substantial negative impacts on plant growth and development (Carrow, 1996; Crasta and Cox, 1996; Faver et al., 1996). This decline in plant growth and development under water deficit is partly because of a decline in the essential nutrient element demand which serve a variety of important metabolic functions (Edeoga et al., 2006). Nutrient uptake by crop plants is generally decreased under water-

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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.

Mannitol (mM)	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	7.24±0.36^a	6.00±0.24^b	6.37±0.27^b	2.56±0.19^a	2.55±0.18^a	2.78±0.21^a	7.38±0.37^a	6.82±0.31^b	7.63±0.39^a	0.00±0.00^i	0.00±0.00^i	0.00±0.00^i
50	7.14±0.32^a	5.64±0.22^c	5.86±0.24^c	2.46±0.17^{ab}	2.28±0.17^b	2.48±0.18^{ab}	7.14±0.32^a	6.50±0.29^b	7.35±0.37^a	5.20±0.22^g	7.28±0.53^g	8.23±0.72^g
100	6.25±0.28^b	5.21±0.21^c	5.22±0.21^c	1.55±0.15^{bc}	1.34±0.13^c	1.28±0.13^c	6.65±0.29^b	5.84±0.27^c	5.98±0.29^c	11.2±1.04^f	16.5±1.82^f	15.8±1.38^f
150	4.23±0.18^d	2.88±0.19^e	2.55±0.19^e	1.34±0.14^c	1.25±0.12^c	1.00±0.12^d	5.65±0.26^c	4.62±0.23^d	4.54±0.27^d	52.5±4.36^d	44.8±4.01^e	43.6±3.96^e
200	2.22±0.19^e	0.24±0.04^g	1.44±0.14^g	0.78±0.10^e	0.65±0.9^e	0.55±0.08^e	4.52±0.21^d	3.55±0.20^e	3.82±0.21^e	62.8±6.56^c	59.5±5.12^d	58.5±5.04^d
250	1.08±0.12^f	0.21±0.03^g	0.37±0.53^g	0.58±0.07^f	0.26±0.03^f	0.24±0.02^f	0.54±0.08^f	0.24±0.02^f	0.35±0.10^f	86.8±8.97^b	78.2±7.58^b	79.3±8.04^b
300	0.54±0.08g	0.00±0.00^h	0.00±0.00^h	0.20±0.01^g	0.00±0.00^g	0.00±0.00^h	0.25±0.04^f	0.00±0.00^g	0.00±0.00^g	65.9±6.58^c	100±9.98^a	100±9.92^a

Significant differences, according to Tukey's HSD test, between treatments are indicated $P\leq 0.05$.

Table 3. Effect of different concentrations of mannitol on fresh weight, dry weight, chlorophyll contents and relative water content 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.

Mannito(mM)	Fresh weight/ five explants (g)			Dry weight/five explant (g)			Chl content [SPAD]			Relative Water Content (%)		
	BM	BT	BS	BM	MT	BS	BM	MT	BS	BM	MT	BS
0.0	2.95±0.25^a	2.83±0.24^b	2.89±0.25^a	1.25±0.13^a	1.22±0.12^{ab}	1.24±0.12^a	65.72±7.25^a	60.34±6.42^b	60.42±6.44^b	81.67±8.58^b	86.56±8.75^a	87.47±8.78^a
50	2.82±0.24^b	2.81±0.23^b	2.88±0.24^b	1.28±0.13^a	1.20±0.12^{ab}	1.30±0.14^a	64.62±7.04^a	60.32±6.35^b	60.23±6.22^b	80.68±8.35^b	84.68±8.53^a	85.85±8.62^a
100	2.65±0.22^c	2.64±0.22^c	2.68±0.23^c	1.20±0.12^{ab}	1.15±0.12^b	1.14±0.12^b	62.54±6.92^b	59.82±6.12^c	60.05±6.14^b	79.58±7.45^c	80.78±8.37^b	82.28±8.25^{ab}
150	2.43±0.18^d	2.45±0.19^d	2.35±0.18^d	1.18±0.12^b	0.88±0.08^c	1.08±0.11^b	60.65±6.50^b	58.28±5.75^c	59.54±6.05^c	79.54±7.25^c	78.22±7.65^c	79.35±7.22^c
200	2.05±0.17^e	2.00±0.16^e	2.08±0.17^e	1.09±0.11^b	0.88±0.08^c	1.02±0.10^b	58.65±6.08^c	56.45±4.25^c	57.24±4.75^c	74.58±7.02^d	73.28±6.92^d	74.48±7.01^d
250	2.05±0.17^e	1.05±0.11^f	1.25±0.13^f	1.05±0.11^b	0.32±0.05^e	0.48±0.06^e	44.37±3.25^d	38.68±3.02^e	42.27±3.18^d	69.85±6.59^e	69.75±6.52^e	68.82±6.32^e
300	1.84±0.15^f	0.00±0.00^g	0.00±0.00^g	1.00±0.10^d	0.00±0.00^f	0.00±0.00^f	37.50±2.88^e	00.00±0.00^f	00.00±0.00^f	67.85±6.65^e	00.00±0.00^f	00.00±0.00^f

Significant differences, according to Tukey's HSD test, between treatments are indicated $P\leq 0.05$.

Table 4. Effect of different concentrations of mannitol (mM) on mineral composition (mg L^{-1}) of *in vitro* micoshoots of *Ficus carica* L cv. Black Mission, Brown Turkey and Brunswick grown on medium supplemented with 3 mg L-1 BAP and 0.5 mg L-1 2iP after five weeks growth periods.

Cultivars	Mannitol mM	K	P	Ca	Mg	Na	Fe	Zn	Mn	Cu
Black Mission	0	30.57±2.95^{bc}	5.46±0.42^f	6.24±0.52^f	3.88±0.27^g	36.83±3.28^a	4.68±0.33f	2.58±0.18^h	1.42±0.14^{hi}	0.87±0.09^i
	50	28.89±2.75^c	5.23±0.38^f	6.11±0.49^f	3.25±0.22^g	34.38±3.05^b	4.37±0.32f	2.25±0.16^h	1.27±0.12hi	0.82±0.09^i
	100	26.55±2.52^{cd}	4.65±0.32^f	5.65±0.39^f	3.04±0.22^g	31.45±2.98^{bc}	4.15±0.30f	2.05±0.15^h	1.05±0.11hi	0.75±0.06^i
	150	24.58±2.42^{cd}	4.09±0.28^f	5.08±0.35^f	2.43±0.19^h	29.48±2.82^c	4.06±0.29f	1.83±0.13^{hi}	0.87±0.09^i	0.52±0.04^j
	200	22.12±2.08^d	3.57±0.25^g	4.00±0.29^f	2.22±0.15^h	25.36±2.62^{cd}	3.77±0.26^g	1.32±0.12^{hi}	0.89±0.10^i	0.35±0.03^j
	250	12.24±1.35^e	1.12±0.12^{hi}	3.25±0.23^g	1.48±0.14^{hi}	20.29±2.02^d	3.52±0.25^g	1.27±0.12^{hi}	0.58±0.05^j	0.26±0.02^j
	300	8.69±0.62^{ef}	0.75±0.08^i	1.12±0.12^{hi}	0.52±0.04^j	12.58±1.09^e	2.23±0.15^h	0.46±0.03^j	0.32±0.02^{jk}	0.09±0.01^k
Brown Turkey	0	31.25±2.98^{ab}	5.58±0.44^f	6.38±0.58^f	3.79±0.26^g	34.38±3.04^a	4.65±0.34^{fg}	2.42±0.19^h	1.68±0.15^{hi}	0.91±0.10^i
	50	28.87±2.76^b	5.29±0.39^f	6.21±0.51^f	3.47±0.24^g	31.78±3.00^{ab}	4.25±0.30^{fg}	2.26±0.16^h	1.28±0.12^{hi}	0.80±0.09^i
	100	26.47±2.60^c	5.15±0.36^f	5.75±0.41^f	2.68±0.20^g	28.49±2.60^b	4.03±0.29^{fg}	1.88±0.13^{hi}	1.12±0.11^{hi}	0.68±0.05^i
	150	24.22±2.38^c	4.68±0.33^{fg}	5.57±0.40^f	2.46±0.19^h	26.48±2.60^c	3.77±0.25^g	1.72±0.15^{hi}	0.82±0.09^i	0.49±0.04^i
	200	20.43±2.03^d	4.05±0.29^{fg}	3.07±0.22^g	1.64±0.14^{hi}	24.88±2.41^c	3.14±0.22^g	0.82±0.09^i	0.38±0.03^i	0.29±0.03^i
	250	11.29±1.07^e	1.62±0.14^{hi}	2.18±0.15^h	0.74±0.06^i	11.44±1.08^e	2.09±0.16^h	0.35±0.02^i	0.26±0.02^i	0.21±0.02^i
	300	0.00±0.00^k	0.00±0.00^k	0.00±0.00^k	0.00±0.00^k	0.00±0.00^k	0.00±0.00^k	0.00±0.00^k	0.00±0.00^k	0.00±0.00^k
Brunswick	0	30.48±2.92^b	5.66±0.45^e	6.00±0.47^e	3.77±0.25^f	35.25±3.05^a	4.88±0.36^{ef}	2.85±0.21^{fg}	1.68±0.14^g	0.94±0.10^h
	50	28.25±2.72^{bc}	5.58±0.44^e	5.98±0.40^e	3.62±0.24^f	32.44±3.02^{ab}	4.27±0.31^{ef}	2.78±0.22^{fg}	1.57±0.15^g	0.82±0.09^h
	100	26.15±2.43^c	4.25±0.30^{ef}	5.52±0.43^e	2.87±0.21^{fg}	30.08±3.93^b	3.88±0.30^f	2.34±0.18^{fg}	1.08±0.12^{gh}	0.65±0.05^h
	150	24.48±2.40^{cd}	3.65±0.24^f	4.93±0.34^{ef}	2.57±0.19^{fg}	28.28±2.42^{bc}	2.79±0.22^{fg}	2.00±0.17^{fg}	0.74±0.06^h	0.45±0.04^i
	200	18.87±1.14^d	3.28±0.23^f	3.86±0.30^f	1.65±0.14^g	26.55±2.60^c	2.08±0.14^{fg}	1.78±0.12^g	0.54±0.05^g	0.32±0.03^i
	250	16.79±1.12^d	1.47±0.14^g	1.73±0.15^g	0.51±0.04^h	14.98±1.09^d	1.89±0.13^g	0.36±0.03^i	0.35±0.03^i	0.30±0.02^i
	300	0.00±0.00^j	0.00±0.00j	0.00±0.00^j	0.00±0.00^j	0.00±0.00^j	0.00±0.00^j	0.00±0.00^j	0.00±0.00^j	0.00±0.00^j
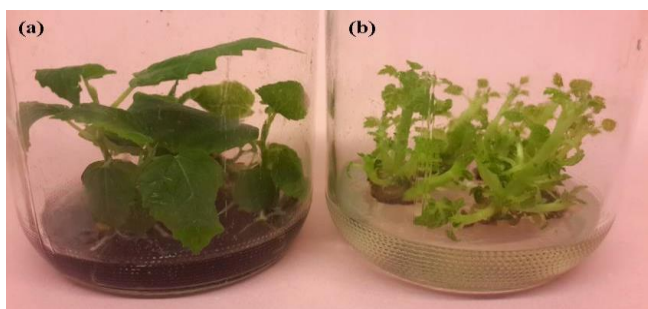
Fig 2. *Ficus carica* cv. Black Mission (a): MS medium supplemented with 3 mg L^{-1}BAP and 0.5 mg L^{-1} 2ip (b): MS medium supplemented with 1mg L^{-1} IBA, 0.5 mg L^{-1} NAA and 2 g L^{-1} activated charcoal (Healthy plantlets with 150 mM mannitol.

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 mM mannitol).

stress conditions owing to a substantial decrease in transpiration rates and impaired active transport and membrane permeability (Levitt, 1980). The plant water status and internal water deficit are related to root system development, and during water stress, root activity and mainly root permeability may change substantially to lower levels. Bartels and Sunkar (2005) generalized the opinion that the capacity of plants to maintain high concentration of K in their tissues seems to be useful trait to take into account in breeding genotypes for high tolerance to drought stress and Ca has been found to regulate the response of the plant to drought.

*Molecular analysis using RAPD and ISSR markers*

Genomic DNA of the fig cvs. Black Mission, Brown Turkey and Brunswick were extracted and the extraction was used in performing Randomly Amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. Three arbitrary oligonucleotide primers were used and the number of fragments 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. These primers amplified 259 PCR bands with RAPD marker and 208 PCR bands with ISSR markers (Tabl 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 figures 4 and 5. The OPAAD-15 and HB15 primer recorded the highest percentage

polymorphism (29.3%; 48%) and revealed 27 and 31 polymorphic bands in 92 and 64 amplified fragments, respectively. In RAPD analysis the Tube O-06 primer recorded the lowest percentage polymorphism (22.5%) and it revealed 18 polymorphic bands in 80 amplified fragments, while in ISSR analysis the lowest level of polymorphism (6.4%) was recorded for the HB11 primer. A high percentage of band polymorphism was arbitrary, while 4 bands (3 positive and one negative) in RAPD analysis and 3 bands (2 positive and 1 negative) in ISSR analysis were found to be useful markers related to drought stress (Figure 4 and 5). Primers OPAI-01 and HB15 seemed to be the only ones not to generate molecular marker related to drought stress. The results of the present investigation are in harmony with those of Rashed et al. (2010) and Rania et al. (2007) who detected positive and negative marker for drought and salt tolerance using RAPD and ISSR markers. In other recent studies the use of molecular marker assisted selection enhanced the identification of genotypic tolerance to biotic stress (Agbicodo et al., 2009; Athar and Ashraf, 2009; Armaghan et al., 2013). Also, Lucia et al. (2002) demonstrate that the effectiveness of PCR based molecular marker targeted to environmentally regulated genes indicated useful variation and thus could be used to monitor the impact exerted by adaption to the environment.

Cluster analysis was carried out on three set of marker profiling data based on RAPD, ISSR and combination of RAPD and ISSR. The results based on all the three DNA markers profiles broadly grouped the three cultivars in two clusters (data not showed). The first cluster had the Black Mission cultivar in RAPD and RAPD+ISSR marker, while in ISSR marker the first cluster had Brown Turkey. In the second cluster which including two sub-clusters varied over different marker systems, one of them maintained the same cultivar, Brunswick in the same sub-cluster, in all three markers system studies and the other sub-cluster contained Black Mission in RAPD and RAPD+ISSR marker or Brown Turkey in ISSR marker. This similarity in the genetic diversity using either RAPD or ISSR approaches between the cultivars in this study was reported also by Wang and Gao (2009). It was evident that there was a close relationship between the cultivars used in this study and they might have been collected from similar locations or may have derived from the same pedigree. Pair-wise similarity index value ranged from 0.765 to 0.839 (RAPD), 0.647 to 0.802 (ISSR) and 0.763 to 0.809 (RAPD+ISSR) and mean similarity index values were 0.802, 0.7355 and 0.786 for RAPD, ISSR and the combined data, respectively (Table 6). In general, considering the genetic divergence between the cultivars, the maximum genetic similarity (0.839) was recorded between Brown Turkey and Brunswick and minimum genetic similarity (765 and 769) were recorded among Black Mission in one side and Brown Turkey or Brunswick in other side, respectively. Thus breeding among Black Mission and Brown Turkey or Brunswick may be useful more than breeding among Brown Turkey and Brunswick in transgressive breeding. Also, the mean similarity index values with RAPD are greater than the similarities observed with ISSR or RAPD+ISSR, which may be due to high polymorphism (Fernandez et al., 2002). Close correspondence between the similarity matrix of RAPD, ISSR and combined RAPD and ISSR was established by means of high value of 0.812, 0.761 and 0.787, respectively. Hence, both the marker systems RAPD and ISSR either individually or combined can be effectively used in determination of genetic relationships among fig cultivars, but RAPD would be a better tool than ISSR for phylogenetic
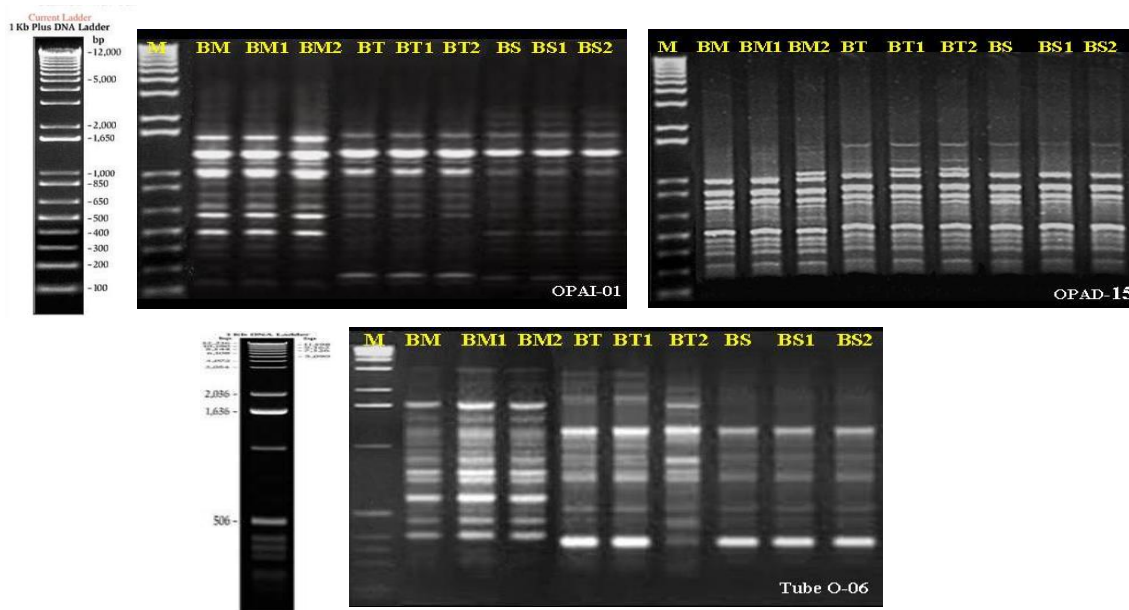
Fig 4. DNA banding pattern generated by RAPD-PCR with different primers (OPAI-01,OPAD-15 and Tube O-06) in *Ficus carica* L cvs. Black Mission, Brown Turkey and Brunswick. Lane M: 1kb plus DNA ladder and 1kb DNA ladder; (BM) Black Mission control, (BM1) Black Mission with 200 mM mannitol, (BM2) Black Mission with 250 mM mannitol, (BT) Brown Turkey control, (BT1) Brown Turkey with 200 mM manitol, (BT2) Brown Turkey with 250 mM mannitol, (BS) Brunswick control, (BS1) Brunswick with 200 mM mannitol and (BS2) Brunswick with 200 mM mannitol.

studies. Saraladevi et al. (2007) indicated that RAPD and ISSR marker systems were found to be useful for the genetic diversity studies and identify variation within cultivars while, Zhang et al. (2013), according to the comparison of genetic cluster analyses, observed that results for RAPD markers were more significant than ISSR markers.

Materials and Methods

The experiments of the present study were conducted in the Tissue Culture and Molecular Genetic Laboratories, Biological Science Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia and Tissue Culture and Biotechnology Laboratories, Marout Research Station, Desert Research Center, Egypt during the period of July 2012 to August 2014.

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 nodal micro-cuttings. The explants employed were shoots of the *Ficus carica* L. cultivars Black Mission, Brown Turkey and Brunswick from previous shoot-tip cultures maintained in MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. Shoots (about 1 - 2 cm in length) were sub-cultured every 4 - 5 weeks in 250 cm^3 jars containing 40 cm^3 of MS solid medium supplemented with 3% sucrose, 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} 2iP as described by Metwali et al., (2014). The pH of the medium was adjusted to 5.8 using 0.1N NaOH or HCl as required 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 fig shoots were sub-cultured on MS medium supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} 2iP Metwali et al., (2014) with different concentrations of Mannitol (Sigma Aldrich) at 0.0, 100, 150, 200, 250 and 300 mM L^{-1}, respectively to study the effect of osmotic stress. Mannitol was added to media before pH adjustment then the osmotic stress level was measured in all media using an 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 minutes with distilled water to remove and culture medium then dried on filter paper and either used to measure the different growth traits or stored at -20°C for later use in molecular studies.

Data measurement

Relative Water Content (RWC)

For the measurement of RWC, fresh leaves were taken from each cultivar after the multiplication stage and weighed immediately to record fresh weight (FW). Then they were placed in distilled water for 4 h and then weighed again to record turgid weight (TW), then 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.

Table 5. Polymorphism rate for Ficus carica L cvs. Black Mission, Brown Turkey and Brunswick using RAPD and ISSR primers.

Markers	Primer code	Sequence (5́----------3́)	Number of bands	Number of Polymorphic markers	Polymorphism (%)
RAPD	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	
ISSR	HB 11	5'- GTGTGTGTGTGTCC-3'	78	5	6.4
	HB 15	5'-GTGGTGGTGGC-3'	64	31	48.4
	844A	5'-CTCTCTCTCTCTCTCTAC-3'	66	11	16.7
Total			208	48	
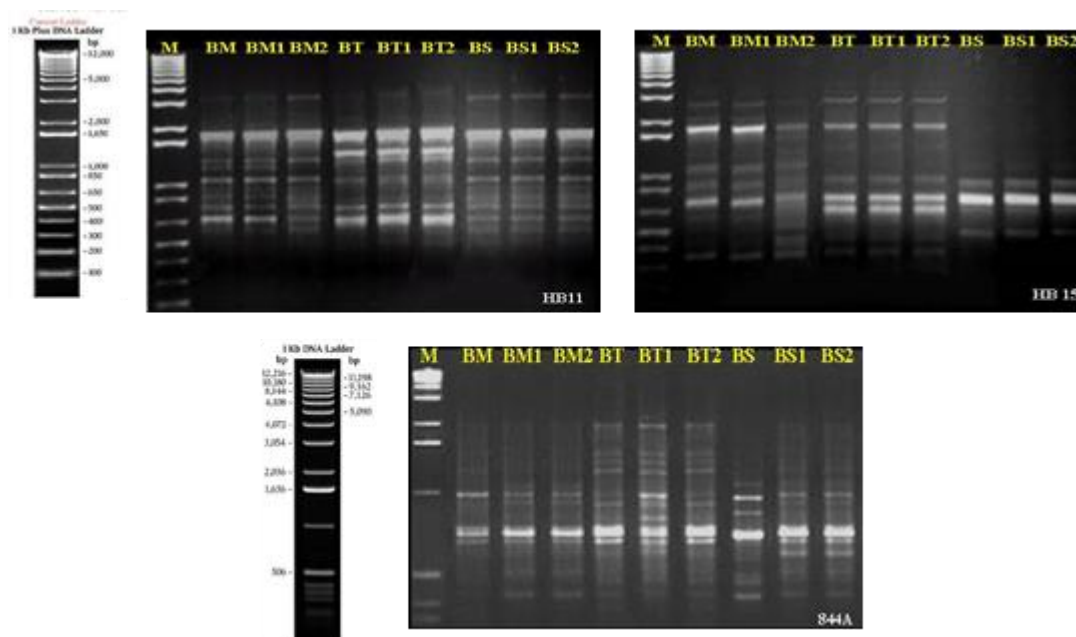
Fig 5. DNA banding pattern generated by ISSR-PCR with different primers (HB11, HB15 and 844A) in Ficus carica L cvs. Black Mission, Brown Turkey and Brunswick. Lane M: DNA marker (1kb plus DNA ladder and 1kb DNA ladder); (BM) Black Mission control, (BM1) Black Mission with 200 mM manitol, (BM2) Black Mission with 250 mM manitol, (BT) Brown Turkey control, (BT1) Brown Turkey with 200 mM manitol, (BT2) Brown Turkey with 250 mM manitol, (BS) Brunswick control, (BS1) Brunswick with 200 mM manitol and (BS2) Brunswick with 200 mM manitol

Chlorophyll (Chl) content

Chorophyll content was measured with a portable leaf chlorophyll meter (*SPAD 502*, Japan).

Mineral composition

Plant samples were oven dried at 80°C for 24 h and ground using a pestle and mortar for subsequent determination of mineral composition according to El-Wanis et al., (2012). The concentration of Na and K were determined by flame emission spectrophotometry according to Jackson (1958), while Ca, Mg, Fe, Zn, Mn and Cu concentration were determined by flame absorption spectrophotometer according to Cheng and Bray (1951). Phosphorus was determined colorimetrically using the ammonium phosphor vanadomolybdate method according to Jackson (1958).

Molecular analysis using RAPD and ISSR marker
Genomic DNA extraction

DNA was extracted from leaf tissue from each cultivar using a cetyl trimethyl ammonium bromide (CTAB) method according to Maniatis et al., (1982). One gram of frozen fig leaves from each entry was ground in a pre-cooled pestle and mortar with 1 mL buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0.2% PVP40 (w/v), 0.2% (v/v) 2-mercaptoethanol), mixed, transferred to a 500 μL microfuge tube and incubated at 65 ºC for one hour. After incubation the mixture was centrifuged for 20 minutes at 4000 rpm at room temperature. The supernatant was taken and RNAse 1:1000 dilution of RNAse (100 mg mL^{-1}) was added and kept at 37 ºC for 30 minutes, 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 overnight at 4ºC. The supernatant was removed from the tube and the pellet was washed with wash buffer (70 % ethanol), centrifuged again for 10 minutes at 1000 rpm at 20ºC and the resulting pellet dried under vacuum. The DNA pellet was re-suspended in 100 μL of deionized H$_2$O and incubated at 50ºC for 15 minutes, centrifuged for 5 minutes at 1000 rpm at 20ºC then the solution was transferred to a new microfuge tube. The DNA quantity was estimated spectrophotometrically by measuring absorbance at 260 nm.

7

Polymerase Chain Reaction (PCR)

After checking the concentration of genomic DNA by agarose gel electrophoresis for all fig cultivars two PCR-based techniques, RAPD and ISSR, were used to detect markers 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 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 Cetus, 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. The data were analyzed using locus-to-locus gel readings, and the rates of *in-vitro* DNA polymorphism were calculated and given as percentage of the total number of bands for the fig plants. The data obtained by scoring the RAPD and ISSR profiles with different primers individually as well as collectively were subjected to the construction of similarity matrix using Jaccard's coefficients (Jaccard, 1908). Data analysis was carried out using SPSS statistical analysis program (software version 10). Using genetic similarity matrices, a dendrogram was constructed based on an un-weighted pair-group method with arithmetic average (UPGMA). The product-moment correlation (r) based on Mantel Z-value (Mantel, 1967) was computed to measure the degree of relationship between similarity index matrices produced by any two-marker systems.

Statistical differences

The statistical analysis was performed using one-way ANOVA and the significance of differences among treatment means were contrasted with Tukey's Honestly Significant Difference Test (HSD) at $P < 0.05$. The program STATISTICA (StatSoft, Tulsa, OK, USA) version 6.0 was used.

Conclusion

In the present study, all growth and physiological traits studied showed a progressive and consistent decrease with the increase in drought stress induced by increased mannitol concentration in the culture medium and a consequent rise in the percentage of necrosis. Drought-stress caused by 300 mM mannitol killed cultivars Brown Turkey and Brunswick but Black Mission survived indicating that this cultivar was the most tolerant. It was evident that RAPD and ISSR assays produced valuable data that could be useful to breeders who can then select related or unrelated parental germplasm to maximize variability in fig breeding programmes under abiotic stress. Genetic relatedness can be done using either or both RAPD and ISSR molecular marker data, dendrograms revealed that the drought tolerance markers revealed close genetic relationships between the three cultivars tested. According to the results in these studies we advise researchers to select several marker methods to obtain information on DNA variability when studying the genetic diversity of fig germplasms as the resulting clustering analysis will be more accurate with this approach.

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