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Response of *Moringa oleifera* Callus and Plantlets to Mannitol-induced Drought Stress.

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IN THIS work, we generated callus and micropropagated plants from *Moringa oleifera* to evaluate their response to drought stress induced by different concentrations of mannitol. Mannitol-induced drought stress caused a reduction shoot and root length as well as fresh and dry weights of shoots and roots in micropropagated plants. In addition, chlorophyll (a and b) and carotenoids contents were reduced. Such a reduction was concomitant with increases in soluble sugars, proline, malondialdehyde (MDA), ascorbic acid (ASA), reduced glutathione (GSH) and total phenols content in both callus and micropropagated plants. Moreover, the activities of catalase (CAT), superoxide dismutase (SOD), polyphenol oxidase (PPO) increased in micropropagated plants parallel with decreases in peroxidase (POX), ascorbate peroxidase (APX) and ascorbate oxidase (ASO) activities. On the other hand, the activities of CAT, SOD, POX, PPO, ASO increased in callus coupled with a decrease in APX. Our data indicated that micropropagated plants are more sensitive to drought stress, compared to callus as evidenced by greater accumulation of MDA while callus accumulated more soluble sugar, proline, GSH and total phenols than micropropagated plants.

Keywords: *Moringa oleifera*, Callus, Micropropagation, Drought stress, Abiotic stress, Mannitol.

Abbreviations : MDA: Malondialdehyde, ASA: Ascorbic acid, GSH: Reduced glutathione, CAT: Catalase, SOD: Superoxide dismutase, PPO: Polyphenol oxidase, POX: Peroxidase, APX: Ascorbate peroxidase, ASO: Ascorbate oxidase, MS: Murashige and Skoog, SIM: Shoot induction medium, SEM: Shoot elongation Medium, 2, 4-D: 2,4-dichlorophenoxyacetic acid , IAA: indole-3-acetic acid, 2-ip: 2-isopentyladenosine, NAA: Naphthaline acetic acid,
IBA: Indole-3-butyric acid, BA: benzyladenine, ROS: Reactive oxygen species.

Abiotic stress limits crop productivity, and plays a major role in determining the distribution of plant species across different types of environments. Recently, the effects of abiotic stress on plants in both natural and agricultural settings

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receive more attention because of the potential impacts of climate change on rainfall patterns, temperature extremes, salinization, and the overall need to maintain or increase agricultural productivity (Boyer, 1982 and Araus *et al*., 2002). Plant biotechnology can help plant breeders by creating and manipulating genetic variability. The contribution of plant biotechnology in plant breeding includes improving both crop quantity and quality (Jauhar 2005).

Water deficit is one of the most important environmental disturbances, which influence the distribution of many species from year to year leading to a tremendous loss all over the world. The tissue culture technique is one of the novel approaches to overcome this problem. The main idea in this respect is that cultivated cells are used as selected units rather than whole plants (Moffat, 1996; Ehsanpour and Amini, 2003). Osmoregulators such as mannitol and sugar alcohols are used to control the osmotic potential in the culture media or nutrient solutions in order to induce water deficit conditions (Zang and Komatsu, 2007).

Moringa oleifera is the best known species of the Moringaceae family (Morton,1991). Moringaceae is a family of plants belonging to order Brassicales; among Angiosperms. It is represented by fourteen species and a single genus *(Moringa*). It is a shrub or small tree which is fast growing, reaching 12 meters in height (Keer and Silva, 1999). It is cultivated for its food, medicinal and culinary value, especially its leaves (Fuglie, 1999). There are tremendous potential opportunities with *M. oleifera* for sustainable agriculture and the development of cash crops in semiarid regions. The few reports on the tissue culture of *M. oleifera* described clonal propagation through the use of nodal explants taken from non-aseptic sources, either from young seedlings or mature plants (Stephenson and Fahey, 2004; Islam *et al*., 2005 and Marfori, 2010). The preservation of *Moringa spp*. is thus of great concern for biodiversity, ethnobotanical, dietary and pharmacological perspectives.

The aim of the present study is to show the response of *M. olifera* callus and micropropagated plantlets to mannitol-induced drought conditions in an attempt to understand the complex mechanisms of drought stress in plants.

Materials and Methods

Plant materials, medium composition and cultural conditions

Healthy uniform seeds of *M. oleifera* were obtained from the botanic garden, Faculty of Science, Ain Shams University, Cairo, Egypt. Seed coats were removed aseptically and seeds were surface sterilized by immersion in 20% sodium hypochlorite (*v/v*) for 15 min, followed by rinsing three times in sterile distilled water. Seeds were planted aseptically in MS basal medium (Murashige and Skoog, 1962) containing 30 g/l sucrose solidified with 8 g/l agar. The pH was adjusted to 5.8, after which the medium was dispensed at 40 ml each in

culture bottles and sterilized by autoclaving at 121 °C for 20 min. Seed cultures were maintained in the dark at 27 ± 1 °C for 15 days. Upon germination, seedlings were transferred to continuous light at 2000-Lux intensity produced from cool white fluorescent lamps.

Callus induction and callus growth

The seedlings consisting of 3–4 nodes were used in this experiment. Leaf explants were prepared and transferred to MS callus induction medium consisting of MS mineral salts and vitamins supplemented with BAP (4 – 18 μM) and -2,4-D (0.5 – 1.5 μM) containing 3% sucrose Mylene *et al.* (2011) . The pH was adjusted to 5.8 ± 0.02 with1N KOH or 1N HCl before autoclaving at 121^0C for 20 min. Cultures were maintained in a growth chamber set at 25 ± 2 ^{0}C, 70% relative humidity and a 16/8 h (light/dark) photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 2 000-Lux. Five explants were cultured per jar. After eight weeks incubation (subculturing occurred every 2 weeks), the calli were excised and sub-cultured on MS medium and under the same growth conditions with four level concentrations of mannitol (0, 100. 200 and 300 mM). The media were replaced twice during the four week incubation period of the experiment.

Micropropagation studies

Micropropagation studies were carried out with nodal explants. The nodal explants were prepared and transferred to a shoot induction medium (SIM) consisting of MS salts and benzylamino purine (2pi) at 0–20 mg/l and naphthaline acetic acid (NAA) at 0–3 mg/l to determine their effect on multiple axillary shoot formation. The percentage of response in terms of number of shoots per explants and shoot length were recorded after15 days from transferring to SIM medium. The obtained micropropagated shoots were then repeatedly subcultured on MS basal medium supplemented with 4. mg/l BA as shoot elongation Medium (SEM) Saini *et al.* (2013). The cultures were maintained in a sterile growth room with 16 h photoperiod at 25 ± 2 ^{0}C with light intensity of 2000 lux and 65% relative humidity. Rooting was initiated on MS medium supplemented with 3 mg/l indole-3-butyric acid (IBA) under the same conditions.

Effect of mannitol on micropropagated plants

The plantlets were transferred to a hormone free medium supplemented with different concentrations of mannitol (0, 100, 200 and 300.0 mM).The jars were incubated in a growth chamber under fluorescent light and at an ambient temperature of 25 ± 2ºC for four weeks. The medium was replaced every 15 days. The plants were then harvested and the morphological criteria as well as the biochemical and the physiological parameters were assayed.

Extraction and estimation of photosynthetic pigments

The concentrations of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were estimated following the method of Metzner *et al.* **(**1965**)**.

Extraction and estimation of carbohydrates

The total soluble carbohydrates were extracted as described by Homme *et al.* (1992). Soluble sugar was determined spectrophotometrically according to the method of Blakeney and Mutton (1980).

Extraction and estimation of proline

The free proline content was extracted and determine according to the method of Bates *et al.* (1973).

Estimation of Lipid peroxidation

Lipid peroxidation product was extracted in 0.5% (w/v) thiobarbituric acid and 20% (w/v) trichloroacetic acid at 95 ˚C for 30 min. then measured using the spectrophotometric method described by Heath and Packer **(**1968).

Estimation of Reduced glutathione

Reduced glutathione (GSH) was extracted in a solution of 1.0 mM of EDTA with 50 µl $HClO_{4,}$ then measured spectrophotometry (Hissin and Hilf, 1976).

Estimation of Ascorbic acid

Ascorbic acid was extracted with 6% trichloroacetic acid then measured spectrophotometrically (Mukherjee and Choudhuri, 1983**)**.

Estimation of Total phenols content

Total phenols were extracted by methanol and determined by Folin–Ciocalteu reagent assay following the method described by Malik and Singh (1980**)**.

Estimation of Antioxidant enzymes

The antioxidant enzymes were extracted in phosphate buffer, pH 6.8 (Mukherjee and Choudhuri, 1983). The crude extract was used for enzyme assay. Superoxide dismutase activity (SOD EC 1.15.1.1) was determined by measuring the inhibition of the auto-oxidation of pyrogallol using a method described by Marklund and Marklund (1974**)**. Catalase activity (CAT EC 1.11.1.6) was assayed according to the method by Chen *et al.* (2000). Peroxidase (POX EC 1.11.1.7) and polyphenol oxidase (PPO EC 1.10.3.1) activities were assayed according to the method of Kar and Mishra (1976). Ascorbate oxidase activity (ASO EC 1.10.3.3) was measured according to the method of Diallinas *et al.* (1997). Ascorbate peroxidase (APX EC 1.11.1.1) activity was measured according to the method described by Koricheva *et al.* (1997).

Statistical analysis

Data were analyzed statistically by ANOVA (analysis of variance) with subsequent comparison of means by the least significant difference (LSD).

Results

Induction and growth of callus under the effect of mannitol

Calli were initiated on the top surfaces of the explant after three weeks of culture incubation on MS medium supplemented with 2,4-D (0.75µM) and BAP (12 µM) (Figure1, and Table 1). Data included in Table 1 indicates that calli emerged from leaf explants of *M. oleifera* showed the highest percentage of formation (96%). These calli were also found to be characterized with a white color and homogenous texture (Fig. 1). The results of the present investigation showed a reduction in the callus fresh weight associated with increasing compactness and color browning was noticed after 4 weeks treatment with mannitol (Fig.1 and Table 3). The above mentioned symptoms were progressively increased as mannitol concentration increased in MS media as clearly shown in Figure 1.

TABLE 1. Percentage of calli (8 weeks) induced on Murashige & Skoog (MS) medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) and Benzyl aminopurine (BAP). Results are shown as means of 10 replicates ± SE.

2,4-D (µM) / BAP (µM)	0.5	0.75	1.0	1.25	1.5
4	41.0±1.014^{a}	38.0±0.568^{a}	36.6±0.458^{a}	34.5±1.212^{a}	31.4±0.458^{a}
6	56.4±0.651^{b}	55.1±0.624^{b}	52.3±0.458^{b}	47.0±1.101^{b}	44.5±0.655^{b}
8	71.0±0.665^{c}	68.3±0.850^{c}	66.0±1.014^{c}	62.1±0.585^{c}	59.0±0.200^{c}
10	84.4±0.458^{d}	82.2±1.059^{d}	79.0±1.637^{d}	76.5±0.763^{d}	73.0±0.721^{d}
12	94.1±0.451^{e}	96.0±0.987^{f}	93.4±0.472^{f}	91.2±0.233^{fg}	89.5±0.360^{f}
14	93.2±0.655^{e}	96.0±0.700^{f}	92.6±0.264^{f}	88.8±0.435^{ef}	86.3±0.404^{e}
16	97.0±0.251^{f}	95.0±0.361^{f}	94.2±0.964^{f}	92.1±0.900^{g}	90.7±0.907^{f}
18	94.2±0.416^{e}	92.4±0.611^{e}	89.5±1.040^{e}	87.4±0.233^{e}	86.1±0.556^{e}
LSD at 0.05 P	0.8626	1.0639	1.2685	1.0861	0.8103

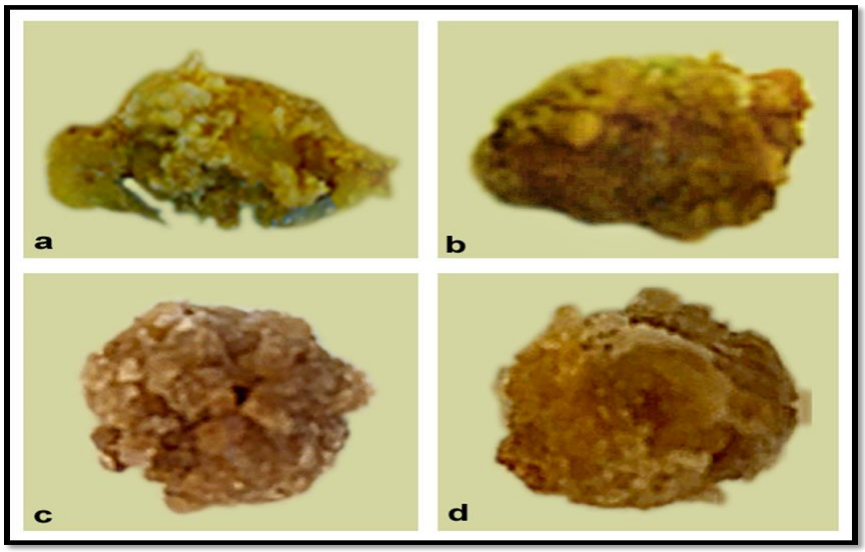

Fig. 1. Callus morphology (12 weeks) incubated with different concentrations of mannitol.
Control (a) with no mannitol, b: 100, c: 200, d: 300 mM mannitol for 4 weeks.

Micropropagation

Shoot micropropagation was obtained from the nodal segments developed from germinated plants under aseptic conditions. Table 2 shows that the cultured shoots length reached about 8 cm with 4-5 internodes and leaves accompanied by a well appearance after five weeks growth in shoot induction medium (SIM) containing 18 mg/l 2 ip and 2 mg/l NAA and 2 weeks shoot elongation medium (SEM) containing 4 mg/l BAP. The well-developed and healthy shoots were transferred to root induction medium (RIM) containing 3 mg/l IBA. Roots with 3-4 laterals were induced by the end of the first week. All the micropropagated plantlets were morphologically similar and also resembled their respective mother plants.

Effect of different concentrations of mannitol on callus and micropropagated plantlets growth

Growth rate of both callus cells and the micropropagated platelets decreased through the 4 weeks of subculturing on MS medium containing different levels of mannitol. Results included in (Table 4, Fig. 2) show a significant decrease in the growth rate parameters as mannitol concentration increased in the culture media. The effect of mannitol on callus cells was more pronounced at 300 mM mannitol followed by 200 and 100 mM, respectively. Although there was a difference in growth between the control and the mannitol-treated cells, there was no significant differences between different levels of treatments. However, the values of fresh and dry weights were progressively decreased with increasing mannitol concentration (Table 4). In general, the results obtained showed that, addition of mannitol caused a reduction in shoot and root elongation rates as well as the fresh and dry weights.

TABLE 2. Effect of different concentrations of naphthalene acetic acid (NAA) and 2 isopentyle adenine (2 ip) (mg/L) on the percentage of micropropagated (5 weeks) plantlets formation. Results are shown as means of 10 replicates ± SE.

NAA / 2ip	0	2	4	6	8	10	12	14	16	18	20
0	16.2±0.66^{a}	19.5±0.31^{a}	24.4±0.31^{a}	27.1±0.46^{a}	30.6±0.35^{a}	34.2±1.02^{a}	41.0±0.51^{a}	49.3±1.17^{a}	52.5±0.61^{a}	58.7±0.96^{a}	64.0±0.53^{a}
0.5	25.9±0.55^{b}	28.4±0.40^{b}	31.5±0.55^{b}	34.1±1.10^{b}	38.5±0.32^{b}	44.0±0.95^{b}	49.3±2.16^{b}	52.5±0.61^{b}	58.7±0.96^{b}	64.0±0.53^{b}	70.1±1.47^{b}
1.0	35.6±0.87^{c}	39.7±0.53^{c}	42.0±0.81^{c}	46.6±0.85^{c}	51.9±0.31^{c}	56.2±1.17^{c}	61.0±1.78^{c}	65.6±0.78^{c}	70.8±1.17^{c}	76.0±0.45^{c}	78.0±0.53^{c}
1.5	54.2±0.52^{d}	57.3±0.46^{d}	60.2±0.36^{d}	63.1±0.42^{d}	68.6±1.09^{d}	71.3±0.62^{d}	73.0±0.78^{d}	75.5±0.84^{d}	79.4±1.83^{d}	81.6±0.31^{d}	82.8±2.14^{e}
2.0	66.7±0.70^{e}	68.4±0.86^{e}	71.8±0.10^{e}	74.2±0.70^{e}	76.8±0.67^{e}	78.4±0.87^{e}	82.9±1.07^{e}	84.2±0.31^{e}	85.4±1.25^{e}	86.7±1.31^{f}	84.9±0.75^{e}
2.5	70.5±0.36^{f}	72.8±0.36^{g}	75.0±0.32^{g}	77.9±0.46^{f}	79.6±0.70^{f}	81.5±1.09^{ef}	83.8±0.89^{e}	85.1±0.95^{e}	86.3±1.01^{e}	85.8±0.79^{ef}	84.1±0.28^{e}
3.0	68.0±0.45^{e}	70.1±0.46^{f}	72.7±0.91^{f}	74.3±0.97^{e}	77.6±0.34^{e}	79.4±0.50^{e}	81.7±0.94^{e}	83.9±0.26^{e}	85.2±0.49^{e}	84.2±0.49^{e}	83.4±1.03^{e}
LSD at 0.05 P	0.86	0.72	0.78	1.06	0.86	1.30	1.82	1.08	1.59	1.08	1.60

TABLE 3. Callus fresh weight (g) incubated on Murashige &Skoog (MS) medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) and Benzyl aminopurine(BAP) as growth regulators. Results are shown as means of 10 replicates ± SE.

2,4-D (µM) / BAP	0.5	0.75	1	1.25	1.5
4	0.34±0.008[a]	0.32±0.004[a]	0.31±0.003[a]	0.29±0.010[a]	0.26±0.003[a]
6	0.47±0.005[b]	0.46±0.005[b]	0.44±0.003[b]	0.39±0.009[b]	0.37±0.005[b]
8	0.59±0.005[c]	0.57±0.006[c]	0.55±0.008[c]	0.52±0.004[c]	0.49±0.011[c]
10	0.70±0.003[d]	0.69±0.008[d]	0.66±0.013[d]	0.64±0.006[d]	0.61±0.006[d]
12	0.78±0.003[e]	0.80±0.007[f]	0.78±0.003[f]	0.76±0.004[f]	0.75±0.003[f]
14	0.78±0.005[e]	0.80±0.005[f]	0.77±0.002[f]	0.74±0.003[e]	0.72±0.003[e]
16	0.81±0.002[f]	0.79±0.003[f]	0.79±0.008[f]	0.77±0.006[f]	0.76±0.007[f]
18	0.79±0.003[e]	0.77±0.005[e]	0.75±0.003[e]	0.73±0.002[e]	0.72±0.004[e]
LSD at 0.05 P	0.0072	0.0087	0.0097	0.0093	0.0086

Fig.2. Showing micropropagated plants (4 months) incubated with different concentrations of mannitol a, 0, b, 100 c, 200, d 300 mM mannitol.

TABLE 4. Effect of drought-induced by mannitol on some growth parameters of *Moringa olifera* micropropagated plantlets. Results are shown as means of three replicates ± SE.

Parameter / Mannitol mM	Length (cm)		Fresh weight (g)		Dry weight (g)	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	12.34±0.352^{c}	10.48±0.291^{c}	10.9±0.786^{c}	6.50±0.152^{c}	3.20±0.208^{c}	2.51±0.066^{b}
100	10.85±0.289^{c}	9.74±0.386^{bc}	9.23±0.480^{b}	5.60±0.152^{b}	2.90±0.152^{c}	2.56±0.094^{bc}
200	9.63±0.196^{b}	8.96±0.148^{ab}	8.10±0.264^{b}	5.20±0.152^{b}	1.80±0.208^{b}	2.80±0.076^{c}
300	7.25±0.270^{a}	8.23±0.308^{a}	6.21±0.304^{a}	4.30±0.208^{a}	1.20±0.100^{a}	2.10±0.052^{a}
LSD at 0.05 P	0.399	0.419	0.711	0.244	0.238	0.104

Effect of mannitol-induced drought on pigment contents in micropropagated plants of Moringa olifera

Drought induced by addition of different levels of mannitol significantly decreased Chl a, Chl b and carotenoid contents. Such reduction was positively related to the mannitol concentration (Fig. 3). The reduction in Chls a, b and carotenoid levels were about 3-4 folds at 100 mM mannitol, compared with those of the control. However, at 300 mM mannitol, the reduction in Chl a reached about 6 folds, compared with that of the control, and about 3 folds for both Chl b and carotenoids.

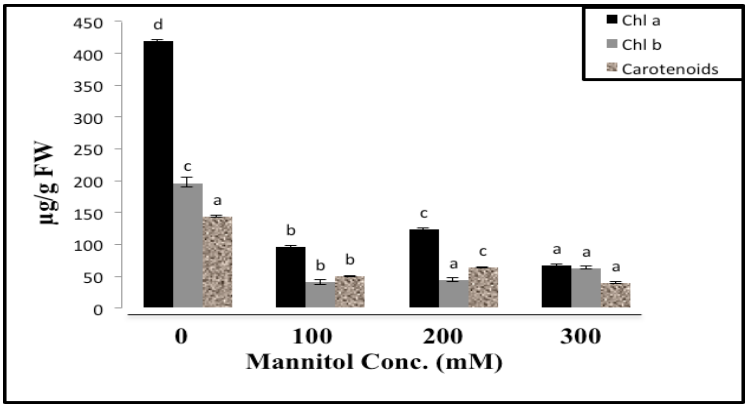

Fig.3. Effect of mannitol induced- drought on pigment contents in micropropagated plants of *Moringa olifera*. Results are shown as means of three replicates ± SE. The LSD at 0.05% level is 2.303, 24.466 and 1.171 in Chl a, b and carotenoids respectively.

Effect of mannitol-induced drought on soluble sugar, free proline and malondialdehyde in micropropagated plants and callus cells

The total soluble sugar concentration in callus increased proportionally with increasing mannitol in the callus medium (Fig. 4A). In case of micropropagated plantlets, the greatest level of soluble sugar was displayed with 100 mM mannitol. However, no difference in the total soluble sugar contents were detected at 300 mM mannitol and the control. Malonaldehyde concentration increased in micropropagated plantlets with increasing mannitol concentration in a higher pattern than callus (Fig. 4 B).

Furthermore, increasing mannitol concentrations significantly increased the accumulation of free proline in callus cells as well as micropropagated plants (Fig. 4C). The increment in the accumulation of proline was more pronounced at 300 mM followed by 100 mM mannitol.

Effect of mannitol-induced drought on ascorbic acid, glutathione and total phenols in micropropagated plants and callus cells

Figure 5 shows that ascorbic acid and total phenols increased significantly by mannitol treatments. The increment in the levels of ascorbic acid reached about 4.1, 3.8 and 3.4 folds at 100, 200 and 300 mM mannitol respectively compared to control (Fig. 5A). However, GSH increased by 1-7% of the control with increasing mannitol levels (Fig. 5B). Meanwhile, the increase in total phenols was about 96, 127 and 174% at 100, 200 and 300 mM mannitol, respectively compared to control (Fig.5C).

In micropropagated plantlets, ascorbic acid was increased by 154, 165 and 126 % compared with the control. The maximum increase in ascorbic acid was measured in the plantlets exposed to 100 mM mannitol, whereas GSH levels increased by 1-4% of the control. Moreover, the total phenols level reached about 129, 151 and 139% at100, 200 and 300 mM mannitol, respectively compared to control (Fig. 5).

Effect of mannitol-induced drought on antioxidant enzymes in micropropagated plants and callus cells

The activities of the antioxidant enzymes exhibited differential response patterns to drought induced by mannitol as shown in Fig. 6. In callus, some enzyme activities slightly increased like CAT or decreased like APX, POX and ASO, while, PPO increased only at 100 and 200 mM mannitol. Among others, increment in SOD activity was more pronounced.

In micropropagated plantlets, some enzyme activities including CAT and APX showed similar responses as those in callus. But, SOD activity increased compared with that in callus. However, some enzyme activities responded differently like POX and ASO as they increased to some extents compared to their activities in callus.

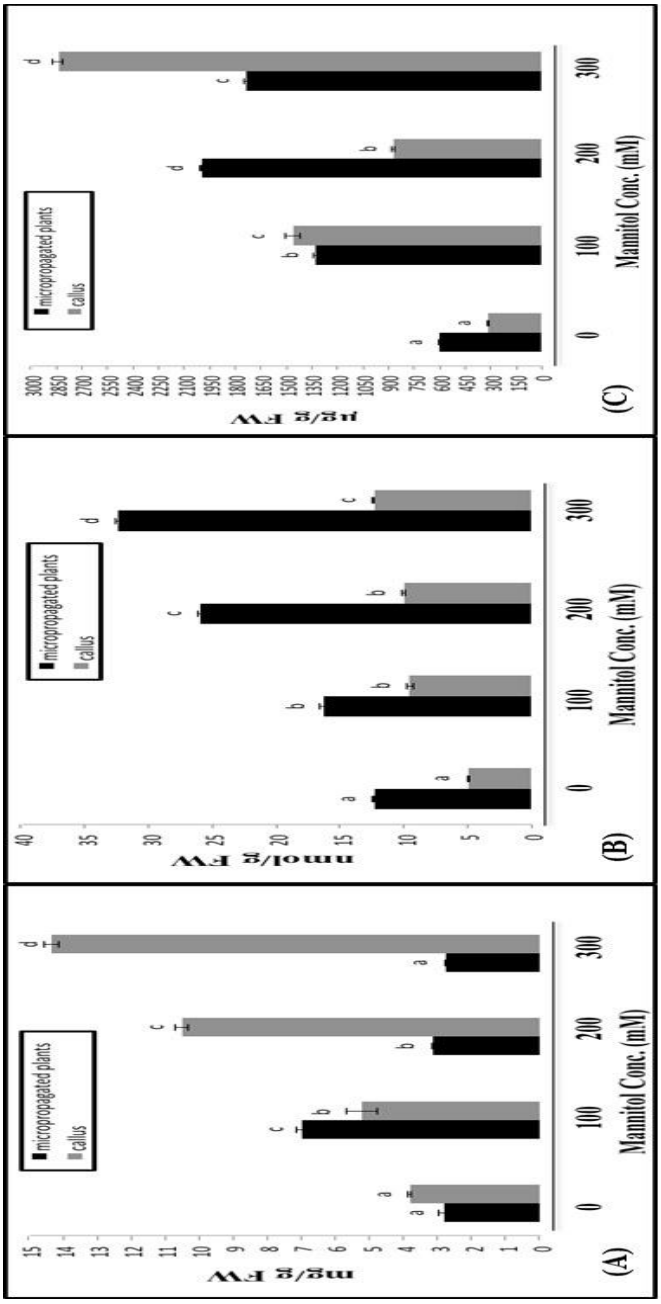

Fig. 4. Effect of mannitol-induced drought on soluble sugar (A), MDA (B) and proline (C) contents in micropropagated plants and callus cells. Results are shown as a mean of three replicates ± SE. The LSD at 0.05% level is 0.125 and 0.372 in (A), 0.299 and 0.184 in (B) and 20.476 and 34.302 in (C) in micropropagated plants and callus cells, respectively.

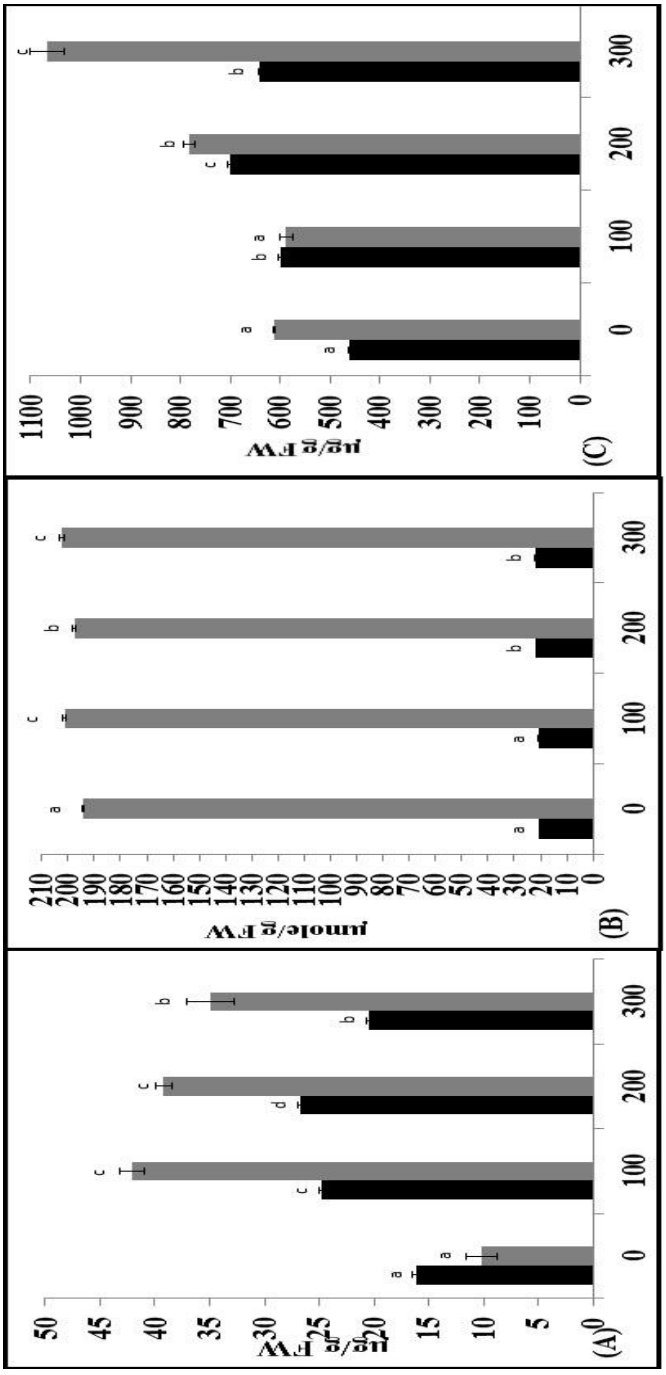

Fig. 5. Effect of mannitol-induced drought on ascorbic acid (ASA) (A), reduced glutathione (GSH) (B) and total phenols (C) contents in micropropagated plants and callus cells. Results are shown as means of three replicates ± SE. The LSD at 0.05% is 0.456 and 2.087 in (A), 0.078 and 0.740 in (B) and 9.827 and 27.114 in (C) in micropropagated plants and callus cells, respectively.

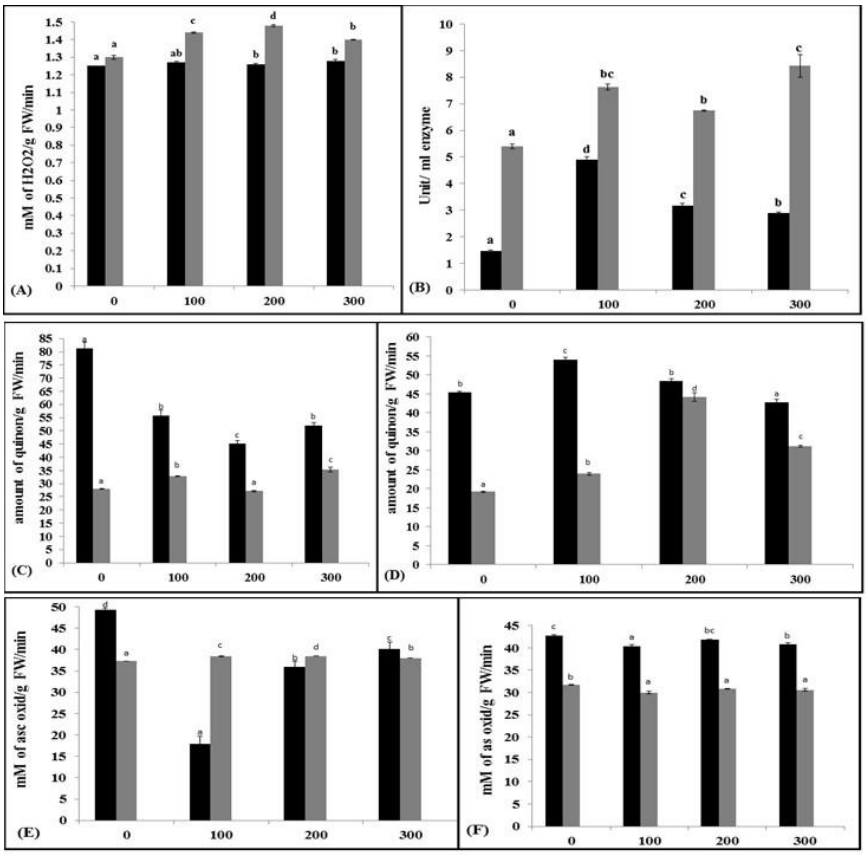

Fig. 6. Effect of mannitol-induced drought on catalase (CAT) (A), superoxide dismutase (SOD) (B) peroxidas (POX) (C), polyphenol oxidase (PPO) (D), ascorbate oxidase (ASO) (E) and (ascorbate peroxidase) APX (F) enzyme activities in micropropagated plants and callus cells of Moringa. Results are shown as a mean of three replicates ± SE. The LSD at 0.05% is 0.007 and 0.009 in (A), 0.110 and 0.325 in (B) and 2.366 and 0.704 in (C), 0.764 and 0.854 in (D), 1.885 and 0.091 in (E), 0.347 and 0.288 in (F) in micropropagated plants and callus cells respectively.

Discussion

Drought stress is one of the major factors limiting the growth of cultivated plants. Drought affects plant physiology and biochemistry and causes a decrease in the efficiency of plant growth and prodcutivity (Arora *et al.*, 2002). The exposure to drought stress induced reduction in the shoot growth (Sankar *et al.*,

2007; Akcay *et al.,* 2010 and Kavas *et al.,* 2013). This reduction was sometimes parallel to inhibition in root growth. In the present study, a decline in root length of *Moringa oleifera* exposed to mannitol-induced drought stress was noticed (Table 4). This might be ascribed to the reduction in cell elongation resulted from reduction in turgor induced by water deficit (Berg and Zeng, 2006). Moreover, our results showed reduction in shoot length during stress conditions at all mannitol levels, which might be attributed to water stress, decreased cell division and enlargement due to low turgor pressure (Manivannan *et al.,* 2007a and Sankar *et al.,* 2007) and these results might indicate a trend of recovery from drought effect with laps of time.

Likewise, drought stress induced by mannitol generally caused reduction in fresh and dry weights of both shoots and roots (Table 4). Devi and Giridhar (2015) reported that drought stress reduced root and shoot fresh and dry weight in Indian soybean varieties. Such increase in root weight provides a relatively large absorption surface and alleviates the stress effect. However, the reduction in dry weight as a result of stress might be attributed to the alteration in carbon and nitrogen allocation and partitioning (Kluge, 1976 and Jaleel, 2009), similar responses in callus tissue of many plants have been shown by Khalequzzaman *et al.* (2005), Sakthiv *et al.* (2008) and Wani *et al.* (2010).

Chlorophyll content in plants is an important factor in determining photosynthetic capacity. The reduction in chlorophyll content is a typical symptom of oxidative stress and one of the adaptation strategies of plants in order to protect the possible photo-inhibition/ photo-dynamic damage during stress conditions (Munne-Bosch and Alegre, 1999).The loss of chlorophyll was attributed to the photoprotection of the photosystem two of light reactions of photosynthesis (Thomas and Stoddart, 1980).

Water deficit induced significant reduction in chlorophyll a and b contents in the present study (Fig. 3). Such effect might be ascribed to loss of chloroplast membranes, excessive swelling, distortion of the lamellae vesiculation, and the appearance of lipid droplets (Kaiser *et al.,* 1981).In this respect, drought stress caused significant decrease in chlorophyll content in leaves of pea (Karatas *et al.,* 2014). Low concentrations of photosynthetic pigments can directly limit photosynthetic potential and hence primary production and/or the production of peroxidative enzymes associated with the degradation of chlorophyll in the thylakoid membrane (Gandul-Kojas *et al.,* 2004).

In plants, the osmotic adjustment contributes to the maintenance of pressure during the drought period. Different compounds play a role in osmotic adjustment such as quaternary amines, amino acids, proline and soluble sugar (Cherian *et al.,* 2006). The present study revealed that the imposition of drought by mannitol significantly increased soluble sugar content in both callus cells and micropropagated plants. The percentage of increase was found to be higher in callus cells than micropropagated plants (Fig. 4A). This result is in

accordance with those of Shehab *et al.* (2010), who reported that drought stress increased the content of soluble sugars in rice (*Oryza sativa*). In fact, soluble sugars serve as important osmoregulators that can adjust osmotic potential and improve stability of proteins. Soluble sugars are not only involved in storage functions but also contribute in controlling cellular metabolism (Harding *et al.*, 2003 and Shao *et al.*, 2005 a, b and c).

Moreover, drought stress negatively affects many aspects of cellular physiology. The major responses to water deficit stress are ROS accumulation, membrane damage and altered antioxidant enzymatic activity, which subsequently lead to the loss of membrane integrity (Li *et al.*, 2011 and Jdey *et al.*, 2014). Lipid peroxidation is considered as a primary cause of membrane oxidative degradation. Malondialdehyde MDA is the product of lipid peroxidation, considered as an indicator of membrane damage (Zhang and Kirkham 1996 and Guo *et al.* 2006). Lipid peroxidation induces free radical as superoxide radical and hydrogen peroxide at the cellular level and may change permeability and composition of membranes. The results of the present investigation showed that, the levels of MDA accumulation was positively related to the extent of mannitol-induced drought stress. Such effect suggests that drought stress caused membrane damage in both micropropagated plants and callus cells. The increments in MDA values were more pronounced in the micropropagated plants than the callus cells (Fig. 4B), which suggested that the formers were more sensitive to drought stress than the callus cells. Shehab *et al.* (2010) observed that drought stress resulted in a markedly increased MDA as a response to elevated oxidative damage promoted by lipid peroxidation under free radical formation.

The accumulation of proline under abiotic stress conditions varied depending on the species and the extent of stress responce. Proline is an osmolyte that plays a diverse role in the reduction of water potential, osmoprotecting, stabilization of proteins, membranes and subcellular structures, and protecting cellular functions by scavenging ROS (Reactive Oxygen Species) (KaviKishor *et al.*, 2005 and Nikolaeva *et al.*, 2010). Proline accumulation was correlated to a variety of stress conditions and is now regarded as a major non-enzymatic antioxidant (Szabados and Savouré, 2010). Proline content increased significantly under drought and severe salt stress conditions in *Ailanthus altissima* seedlings, supporting its role as a protective agent under oxidative stress conditions (De Carvalho *et al.*, 2013). These data was consistent with our results, which showed the accumulation of proline under drought stress condition, particularly in callus cells (Fig. 4C). It was concluded that plants accumulate compatible solutes, such as proline, in response to stresses to facilitate water uptake, protect cells against oxidative

damage and alleviate the negative effect of drought stress on water status and plant growth (Ashraf and Foolad, 2007).

To minimize the effects of oxidative stress, plants have evolved an antioxidant system comprising low molecular antioxidant substances including carotenoids, phenols, ascorbic acid and GSH as well as ROS scavenging enzymes, such as superoxide dismutase, peroxidase and catalase (Apel and Hirt, 2004). Our data show the differential responses in the antioxidant enzyme activities reflecting the different antioxidant metabolism in response to drought stress.

Significant increase in glutathione (GSH) content was recorded in both micpropagated plants and callus cells exposed to mannitol-induced drought stress (Fig. 5B). Glutathione plays a protective role in scavenging singlet oxygen, peroxides and hydroxyl radicals as it is involved in the ascorbate-glutathione pathway in chloroplasts (Foyer, 1993).

In addition, the activities of both superoxide dismutase and catalase were markedly increased in both drought stressed micropropagated plants and callus cells exposed to different levels of mannitol (Fig. 6 A& B).

Superoxide dismutase neutralizes the superoxide radical in the chloroplast and catalyzes the first step in the detoxification of active oxygen forming H_2O_2. Hydrogen peroxide is then scavenged by catalase, resulting in the dismutation of H_2O_2 to water and oxygen. Peroxidase (POX) enzyme plays a vital role in decomposition of H_2O_2 toxicity to water and molecular oxygen (O_2) hence, preventing the cellular damage under water deficit stress (Frederick *et al.,* 2001 and Mohsenzadeh *et al.,* 2006). In the present study, a significant increase in POX activity was measured in callus cells (Fig. 6C). In contrast, the POX activity was decreased in micpropagated plants under drought stress induced by mannitol. This reduction was concomitant with a pronounced increase in the total phenols contents which are substrates for POX and PPO enzymes. Phenolic compounds are antioxidants being able to neutralize the activated and toxic reactive oxygen species (Ksouri *et al.,* 2011).These results are in accordance with those of Karatas *et al.* (2014) who found that drought stress markedly enhanced the activities of SOD, CAT, POX but slightly changed the activity of APX in pea (*Pisum sativum* L.).

Among the non-enzymatic antioxidants, ascorbate is one of the best characterized compounds, required for many key metabolic functions in plant cells (Smirnoff and Wheeler, 2000). In the present study, drought stress induced by mannitol stimulated the production of ascorbic acid content in both micpropagated plants and callus cells (Fig. 5A). The reduction in APX and ASO activities reflected the increases in ASA levels. Ascorbic acid acts as an antioxidant, protecting cells against oxidative stress. ASA has the capacity to eliminate different ROS including singlet oxygen, superoxide and hydroxyl radicals (Foyer, 2001).

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إستجابة كل من كالوس وأكثار نبات المورينجا أوليفيرا لإجهاد الجفاف المستحث بالمانيتول

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الإجهاد اللا حيوى له أنواع مختلفة منها الجفاف، درجات الحرارة القصوى واالملوحة التى بدورها تؤدى إلى نقص فى إنتاجية النبات. التغير المناخى يزيد من الإجهاد اللا حيوى فى العديد من المناطق نتيجه لتغير أنماط هطول الأمطار، درجة الحرارة والرياح. تعتبر زراعة الأنسجة النباتية كآليه للتقنية الحيوية النباتية فى إنشاء صفات وراثية مرغوبة فى الأصناف المختلفة من النباتات لتصبح أكثر مقاومة للإجهاد اللا حيوى وتحسين الإنتاجية. وفي هذا العمل تم إنتاج كالس و إكثار لنبات المورينجا أوليفيرا ودراسة التأثيرات الفسيولوجية العديدة تحت تأثير تركيزات مختلفة من المانيتول كمادة مستحثة للجفاف. ولقد أدى الإجهاد الجفافي إلى نقص فى الأطوال و الأوزان الغضة والجافة لكل من المجموع الخضرى والجذرى للنباتات الناتجة من إكثار المورينجا. ولقد لوحظ أن هناك نقص واضح فى مستويات الأصباغ النباتية (اليخضور) أ و ب و الكاروتيدنات. هذا النقص كان مصحوبا بزيادة واضحة فى محتوى كل من السكريات الذائبة و البرولين و المالونالدهيد و حمض الأسكوربيك و الجلوتاثيون و الفينولات الكلية فى كل من الكالوس و النباتات الناتجة من إكثار المورينجا . ولقد أسفرت النتائج عن زيادة فى بعض أنشطة إنزيمات مضادات الأكسدة كالكتاليز والسوبر أوكسيد ديسموتيز و البولى فينول أوكسيديز مع نقص فى كل من البيروكسيديز و اسكوربيت بيروكسيديز و اسكوربيت اوكسيديز فى النباتات الناتجة من إكثار المورينجا . وعلى الجانب الآخر كان هناك زيادة فى أنشطة إنزيمات كل من الكتاليز و السوبر أوكسيد ديسموتيز و البولى فينول أوكسيديز و البيروكسيديز و اسكوربيت اوكسيديز مصحوبة بنقص فى نشاط اسكوربيت بيروكسيديز فى الكالوس. ولقد أكدت نتائج هذا البحث أن النباتات الناتجة من إكثار المورينجا بطريقة زراعة الأنسجة تكون أكثر حساسية للجفاف من الكالوس المنتج من زراعة الأنسجة كما يتضح من تراكم المالونالدهيد فى النباتات الناتجة من إكثار المورينجا بينما الكالوس تراكمت محتويات كل من السكريات الذائبة و البرولين و الجلوتاثيون والفينولات الكلية.