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Performance of *in vitro* propagated olive (*Olea europea* cv. Manzanillo) under drought stress

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The current study was carried out to investigate the effect of low water potential generated by polyethylene glycol (PEG 6000) on growth of *in vitro* propagated 'Manzanillo' olive cultivar. The response of 'Manzanillo' olive cultivar to *in vitro* multiplication was studied on either Rugini olive medium (OM) and Murshige and Skoog (MS) medium supplemented with three zeatin concentrations, *i.e* 1.25, 2.5 and 5mg L^{-1}). Different levels of water stress were induced using four concentrations of PEG 6000 (0, 25, 50 and 75g L^{-1}). The effect of nutrient media was obvious; 'Manzanillo' growth showed better performance on OM compared with MS. On the other hand, 5mg L^{-1} zeatin recorded the highest shoot number, shoot length and number of leaves. Regarding water stress treatments; survival percentage decreased gradually with increasing PEG concentration in growth media. The evaluation of growth reveals a significant reduction in shoot length, shoot fresh weight, moisture percentage and chlorophyll pigments concentration under drought stress. Water stress increased defoliation percentage and leaf proline content. Protein profile in olive shoots has been changed under drought stress.

Keywords: *Olea europea*, Micropropagation, Water stress, Polyethylene glycol, Protein profile.

INTRODUCTION

Olive tree is well known for its tolerance to severe water stress (Giorio et al., 1999 and Ben Ahmed *et al.,* 2009). Hence the olive cultivation is highly encouraged in arid and semi-arid areas (Loreto et al., 2003); however, tree growth and productivity is affected by water stress (Gargouri et al., 2012). Drought is one of the most important stress condition wide world, it is predicted that climate change and global warming phenomenon will double the drought affected area (Le Houérou, 1996). Drought stress cause significant reduction in plant growth and development (Jain, 2001), and disrupted different physiological processes such as photosynthesis, respiration, ion uptake and metabolism (Wang et al., 2000 and Jaleel et al., 2009).

The conventional breeding programs for tolerance to environmental stress are being used to integrate tolerance genes into the commercial cultivars (Rai et al., 2011). Classic breeding techniques are time consuming and inefficient because of complex genetic nature of tolerance mechanism and lack of well defined selection criteria (Purohit et al., 1998). In addition, it is difficult to analyze plant response to drought stress under field conditions (Lascano et al., 2001). *In vitro* selection for drought stress has been reported in many fruit species, including kiwifruit (Save and Adillon, 1990), common fig (Karimi et al., 2012) and grapes (Duncan et al., 1995). *In vitro* screening will minimize the effect of the external environment (Rai et al., 2011). Polyethylene glycol (PEG) is one of the reliable methods for screening genotypes under water stress (Kocheva and Georgiev 2003; Sakthivelu et al., 2008). PEG has a high molecular weight and nontoxic to plant tissues (Tewary et al., 2000).

The addition of PEG has been used to simulate drought stress that adversely affected the plant growth (Hassan et al., 2004; Gopal and Iwama, 2007). The objective of this study was to evaluate drought tolerance of *in vitro* growing Manzanillo olive plants and to identify possible molecular markers for drought tolerance.

MATERIALS AND METHODS

The current research was carried out during 2015/2016 seasons at the laboratory of Pomology Department, Faculty of Agriculture, Cairo University, Giza, Egypt.

Plant material and culture conditions

Active spring shoots were collected from mature olive trees (*Olea europea*) trees of 'Manzanillo' cultivar (grown at the nursery, of Faculty of Agriculture, Cairo University, Giza, Egypt). Shoots were stripped of leaves, washed with tap water, and divided into nodal cuttings. Surface sterilization was performed with commercial bleach (5.25% sodium hypochlorite) for 10 min, followed by Mercury chloride at 1000 mg L^{-1} for 5 min, and then washed several times with sterile distillated water.

Micropropagation

Olive nodal cuttings were cultured on MS (Murashige and Skoog, 1962) or Rugini Olive media (Rugini, 1984), both of them were supplemented with zeatin (1.25, 2.5 or 5mg L^{-1}), 30 g L^{-1} mannitol and 6.5 g agar L^{-1}. Media pH was adjusted to 5.8 before adding agar and the media was autoclaved at 121°C for 15 min. All cultures were maintained in growth chamber at 25°C and 16h photoperiod (provided from 40-60 µmol $m^{-2}s^{-1}$ cool-white fluorescent lamps). After four weeks, the sprouting percentages were recorded and sprouted buds were transferred to fresh media with the same composition and the sub-culture was performed every four weeks. Proliferation rate, shoot length and number of leaves were recorded at the end of 3rd sub-culture.

Polyethylene glycol treatments

Manzanillo olive shoots of the 3rd subculture were used for PEG treatments, water stress was induced by different osmotic potential levels (0 (control), 25, 50 and 75 g L^{-1} of PEG 6000); the required amount of PEG 6000 was dissolving in OM media only before autoclaving. At the end of experiment (8 weeks), olive shoots were removed from the culture media and gently washed with tap water, and the following parameters were recorded**;** survival percentage, total number of leaves/shoot, defoliation percentage, shoot fresh weight and moisture percentage. Chlorophyll a and b concentration were determined spectrophotometrically using 80% acetone as a solvent (Lichtenthaler and Wellburn, 1983) whereas proline concentration was determined according to Bates et al., (1973).

Polyacrylamide gel electrophoretic analysis

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) to detect the effect of drought treatments on protein profile. Protein fractionation was performed on vertical slab (16.5×18.5×0.2 cm) Hoefer E600, Amersham Pharmacia biotech.

a. Protein extraction

Protein was extracted from leaves of control and PEG treatments. A fresh sample of 0.2 g of leaves was grounded in liquid nitrogen to fine powder. A ratio of 1:2 (w/v) of tissue sample to extraction buffer (0.79 g Tris-HCl, 10µl Bromophenol blue, 2 gm SDS, 10 ml glycerol, 5ml β-mercaptoethanol) was added directly to mortar. Samples were centrifuged at 10,000 rpm at 4oC for 10 min. The supernatants containing water-soluble proteins were transferred to eppendorf tubes and kept at deep-freeze until use.

b. Polyacrylamide gel electrophoresis

A volume of 60 µl protein sample was loaded in a separate well of 12.2% SDS-polyacrylamide gel and control wells were loaded with standard protein marker. SDS-polyacrylamide gel allowed running at constant electric current (200 volt) until the Bromophenol blue dye reached the bottom of the separating gel. Gels were stained by 0.125% Coomassie brilliant blue dye (R 250) overnight. The staining solution was removed and the gels were covered with destining solution of methanol: acetic acid: water (5:1:4). The destaining solution was changed several times until the gel background became clear. Gels were photographed and analyzed using Gel Doc Bio-Rad system.

Statistical analysis

The treatments were arranged in a complete randomized design with three replicates for each treatment, data were subjected to variance analysis (Snedecor and Cochron, 1991) and means were compared according to Duncan's multiple range tests at 1% level (Duncan, 1955).

RESULTS AND DISCUSSION

Micropropagation

Data presented in Table (1) showed that nutrient media and zeatin concentration have a slight effect on sprouting % of Manzanillo olive. Shoots grown on OM medium recorded higher multiplication rate, shoot length and number of leaves compared with MS medium. Increasing zeatin concentration in the growth medium increased olive multiplication rate. The highest multiplication rate was recorded with 5 mgL^{-1} zeatin compared with the other two concentrations. There was an obvious difference between the used zeatin concentrations regarding the shoot length and number of leaves per shoot.

As previously reported, *in vitro* propagation of olive is highly dependent on growth medium (Grigoriadou et al., 2002), cytokinin type and its concentration (Grigoriadou et al., 2002 and Hegazi et al., 2017). The OM medium, MS medium and modified MS medium (Fiorino and Leva, 1986) are the most suitable media for olive micropropagation. The obtained results showed that, nutrient media and zeatin concentration play essential role for *in vitro* propagation of olive. In most cases, zeatin is utilized as a cytokinin for olive; according to Grigoriadou et al. (2002) the highest proliferation rate, number of shoots/explant and shoot height, were obtained with 20 µM zeatin. Rostami and Shahsavar (2012) reported that increasing cytokinin concentration significantly increased number of shoots, shoot length and number of leaves of olive explant.

Polyethylene glycol treatments

Data in table (2) showed that survival percentage significantly decreased with increasing level of PEG. Both control and 25 g L^{-1} PEG treatments recorded 100% survival percentage while 75 g L^{-1} PEG recorded 64%. Concerning growth parameters under water stress, the highest value for shoot length and fresh weight was recorded in control treatment, while 75 g L^{-1} PEG recorded the lowest value.

Data in Table (3) and Figure (1) showed that total number of leaves ranged from 37.33 of control to 25.33 for 75 g L^{-1} of PEG. Increasing severity of water stress generated by PEG increased defoliation percentage from 11%, in the control treatment to 61.67 and 72.33 % at 50 and 75 g L^{-1} of PEG, respectively. Although, moisture content decreased from 90.2% in the control to 75.00%, 74.40% with PEG level 50 and 75 g L^{-1}, respectively. The obtained results showed that drought stress affected the whole plant growth, which may be attributed to the reduction of cell expansion under water deficit (Taiz and Zeiger, 2007). According to Chartzoulakis et al. (1999), stress conditions led to changes in leaf water status. Moreover, drought disrupts most of plant physiological parameters (Boyer, 1982; Giorio et al., 1999).

Table (1) Effect of nutrient media and zeatin concentration on Manzanillo olive.

Media	Sprouting %	Multiplication rate	Shoot length	Number of leaves/ shoot
OM				
zeatin 1.25 mg L^{-1}	69.70^{b}	1.57^{c}	7.500^{d}	10.60^{c}
zeatin 2.5 mg L^{-1}	71.66^{ab}	1.80^{b}	11.83^{b}	13.66^{b}
zeatin 5.0 mg L^{-1}	74.70^{a}	2.40^{a}	14.33^{a}	15.66^{a}
MS				
zeatin 1.25 mg L^{-1}	63.02^{c}	0.84^{e}	4.10^{e}	4.80^{f}
zeatin 2.5 mg L^{-1}	63.20^{c}	0.90^{e}	7.30^{d}	6.10^{e}
zeatin 5.0 mg L^{-1}	64.10^{c}	1.15^{d}	9.80^{c}	8.60^{d}

Means followed by the same letter within each column are not significantly different at $p< 1\%$.

Table (2) The effect of PEG on survival (%), shoot length and shoot fresh weight.

PEG (gL^{-1})	Survival (%)	Shoot length (cm)	Shoot fresh weight (g)
Control	100^{a}	9.66^{a}	1.37^{a}
25	100^{a}	6.33^{b}	0.88^{b}
50	88^{b}	5.00^{c}	0.57^{c}
75	64^{c}	4.00^{d}	0.40^{c}

Means followed by the same letter within each column are not significantly different at $p< 1\%$

Table (3). The effect of PEG on total number of leaves/ shoot, defoliation (%) and moisture (%).

PEG (gL^{-1})	Total number of leaves/ shoot	Defoliation (%)	Shoot moisture (%)
Control	18.67^{a}	5.11^{d}	90.20^{a}
25	15.00^{b}	38.91^{c}	80.00^{b}
50	13.83^{c}	62.47^{b}	75.00^{c}
75	12.67^{d}	72.12^{a}	74.40^{c}

Means followed by the same letter within each column are not significantly different at *p*< 1%.

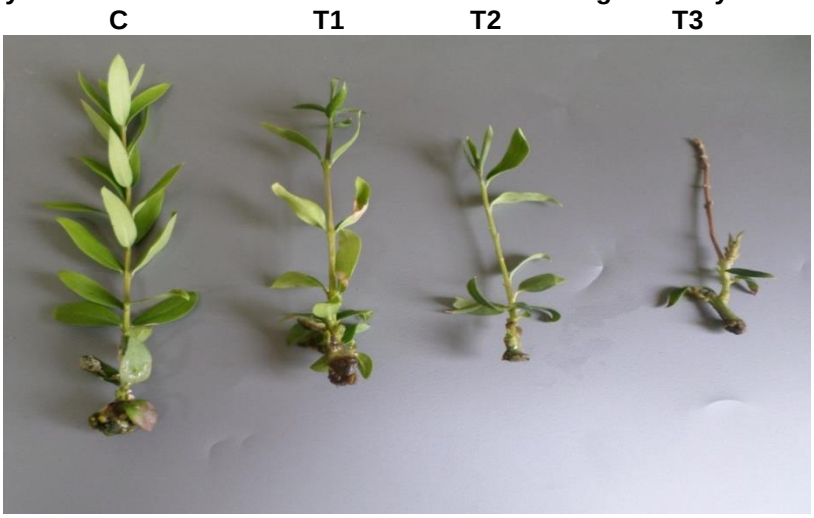

Figure (1) The effects of PEG induced drought stress on growth of Manzanillo olive cultivar, C (Control); T1 (PEG, 25 g L^{-1}), T2 (PEG, 50 g L^{-1}), T3 (PEG, 75 g L^{-1}).

As shown in Table (4) chlorophyll a and b concentration were gradually decreased with increasing PEG in the medium. Although, supplementation of the culture medium with higher concentration of PEG caused a significant increase in total proline content compared with control treatment. The obtained results agree with those reported by Kiani et al. (2008) who reported that drought stress caused a significant reduction of chlorophylls in many plant species. According to Guerfel et al. (2009) a marked reduction in leaf chlorophylls content has been observed in olive plants growing under water stress conditions. Proline is one of the most wide spread compound accumulated in plants in response to water stress (Reddy et al., 2004, Wang et al., 2003 and Yeo, 1998). Proline plays an important role in osmotic adjustment, reduces cellular oxidative damage and maintains water uptake and photosynthetic activity (Hasegawa et al., 2000).

Table (4). Effect of PEG concentration on chlorophyll (*a* and *b*) and proline content.

PEG (g L^{-1})	Chlorophyll *a* (µg g^{-1} FW)	Chlorophyll *b* (µg g^{-1} FW)	Proline (mmol. 100g^{-1} FW)
Control	3.90[a]	6.97[a]	7.42[d]
25	1.27[b]	2.28[b]	7.97[c]
50	0.96[bc]	1.72[bc]	8.29[b]
75	0.75[c]	1.35[c]	8.71[a]

Means followed by the same letter within each column are not significantly different at $p< 1\%$.

Table (5) SDS-PAGE band numbers and banding patterns of Manzanillo cultivar cultured under different PEG concentrations

Band No.	Molecular weight (KD)			
	Lane2	**Lane3**	**Lane4**	**Lane5**
Band1	183.68	87.117	87.117	87.117
Band2	15.258	36.031	36.031	76.69
Band3	-----	15.258	15.258	36.031
Band4	-----	-----	-----	15.258

Polyacrylamide gel electrophoretic analysis.

Data presented in Figure (2) and Table (5) showed the protein pattern of the control treatments (Lane 2) as well as water stress treatments (Lanes 3 to 5). Protein profile of stressed and non-stressed plants showed the presence of five protein types. Protein analysis revealed that, a decline in certain protein (183.68 KD), protein remained unchanged (15.258 KD), and *de novo* production of protein types (87.117, 36.031 and 76.69 KD) occurred under water stress.

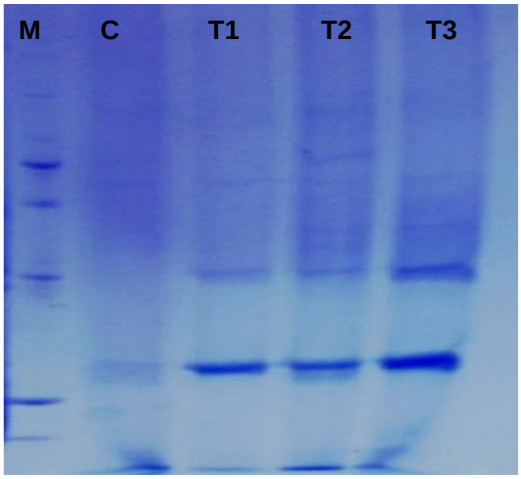

Fig (2). Protein profile of Manzanillo cultivar cultured under PEG treatments, M (Marker); C (Control); T1 (PEG, 25 g L^{-1}), T2 (PEG, 50 g L^{-1}), T3 (PEG, 75 g L^{-1}).

These results suggest that exposing olive to water stress might stimulate expression of genes related drought tolerance. As previously reported, plants face stress conditions by regulating specific sets of genes (Wang et al., 2003). Change in proteins pattern under stress condition have been observed in response to high temperature, salinity and drought (Viswanathan and Khanna-Chopra, 1996). Plomion et al. (1999) suggested that drought stress caused profound alterations in cellular metabolism, such as protein functions. The different banding patterns observed in the current study under different water stress levels indicated that drought tolerance is controlled by several genes (Mitra, 2001).

CONCLUSION

On the basis of the obtained results, it can conclude that, *in vitro* evaluation could be considered as a reliable method for screening genotypes for drought tolerance. PEG has been successfully used to simulate water stress conditions. Moreover, Protein analysis showed a marked changed in plant protein profile under drought stress. Some *de novo* protein types appeared under water stress which may be used as a possible molecular marker for drought tolerance.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest".

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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