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GROWTH BEHAVIOUR OF *IN VITRO* GROWING AGGEZI SHAMI AND PICUAL OLIVE CULTIVARS IN RESPONSE TO BORON STRESS

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Abstract

The olive tree is one of the oldest cultivated plants in the Mediterranean basin and has recently become the largest part of Egypt's agricultural initiatives and projects. *In vitro* micropropagation represents the rapid technique for clonal propagation in order to produce a large number of high-quality plants. Boron (B) is known as an essential micronutrient element for all plants. To date, nothing is known about B deficiency and toxicity of *in vitro* olive culture. The present research was to investigate the response of two *in vitro* olive cultivars to deficiency and toxicity conditions of B in the culture medium. Olive shoots of 'Aggezi Shami' and 'Picual' cvs. were grown on Rugini Olive Media (ROM) supplemented with five B concentrations (0, 12.6 (control), 50, 100 and 150 mg L^{-1} of boric acid). Our results indicated that B deficiency and toxicity had a negative effect on *in vitro* growing olive shoots of both cultivars. Control and 50 mg L^{-1} of B concentration produced higher values of olive shoot length and leaf number. On the contrary, high B concentrations in the culture medium signiûcantly increased shoot number. Olive shoots exhibited a moderate chlorotic appearance and high shoots mortality was recorded with 0 and 150 mg L^{-1} B. No significant differences were detected for shoot fresh weight and proline content of both cultivars under boron stress. By increasing B concentration of the culture medium B contents in explant increased as well as chlorophyll contents declined as B concentration of the culture medium increased.

Key words: olive, micropropagation, boron, toxicity, stress.

Introduction

Olive (*Olea europaea* L.) is one of the most ancient cultivated plants throughout the Mediterranean area in general and in Egypt in particular. Boron (B) is an essential micronutrient element and its role in an array of critical physiological processes is getting expanding consideration. An accurate B concentration in the soil is necessary for the normal plant growth and development (Tanaka and Fujiwara, 2008). The range between B deficiency and toxicity is very narrow (Camacho-Cristóbal *et al*., 2008). B can easily be toxic for plants when its concentration is slightly higher than that required for normal plant growth (Mengel and Kirkby, 2001). B toxicity occurs because of over fertilization and the use of irrigation water with high B content (Branson, 1976; Gupta *et al*., 1976). Application of B-enriched fertilizers is an effective solution for resolving B defi ciency, but B toxicity is a more difficult problem to manage and the damage of B toxicity to plants is irreversible (Leyshon and James, 1993). B deficiency and toxicity affect physiological and biochemical processes, B toxicity symptoms includes; leaves chlorosis, growth inhibition and death of apical meristems and increases oxidative stress (Cervilla *et al*., 2007; Goldbach *et al*., 2001; Han *et al*. 2009; Herrera-Rodríguez *et al*., 2010; Reid and Fitzpatrick, 2009; Sheng *et al*. 2010). Excess B can result in reduced photosynthesis, stomatal conductance and decreased photosynthetic pigments. Moreover, excess B inhibits photosynthesis through alteration of electron transport rate via structural impairing of thylakoids (Landi *et al*., 2013; Lovatt and Bates, 1984; Pereira *et al*., 2000). These physiological disorders arise from B toxicity can be attributed to B-induced oxidative and accumulation of reactive oxygen species (ROS). They induce cell death *via* oxidizing lipids, pigments, proteins and nucleic acids as well as inactivating enzymes (Blokhina *et al*., 2003). Under *in vitro* conditions, B is required for normal shoot growth and development (Nable *et al*., 1997) and is included routinely in all tissue culture media formula. The *in vitro* tissue culture-based techniques allowed the development of new crop improvement tools, including *in vitro* selection through application of selective agent in culture media

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(Sakhanokho and Kelley, 2009; Benderradji *et al*, 2011). The use of tissue culture to evaluate genotypes for resistance to metal toxicities and deficiency has been reported in several crops. Huang and Graham, (1990) concluded that wheat genotypes resistant to B toxicity were also resistant to B toxicity at the cellular level. Similar findings have also been reported for Zn toxicity (Wu and Antonovics, 1978) and iron deficiency (Graham *et al*. 1992; Stephens *et al*., 1990).

The similarities of the effects induced by the stress in both of *in vitro* and *in vivo* conditions suggest that the *in vitro* system can be used as an alternative to field evaluations (Perez-Clemente and Gómez-Cadenas, 2012) and minimize the environmental effect (Rai *et al*. 2011). *In vitro* selection for B toxicity has been reported in kiwifruit (Sotiropoulos and Dimassi, 2004), pear (Sotiropoulos *et al*. 2006 a anb b) and apple (Molassiotis *et al*., 2006; Mouhtaridou *et al*., 2004). To date, our knowledge of B deficiency and toxicity of *in vitro* olive culture is very scarce and needs more studies to understand the morphological and physiological responses to B toxicity/tolerance. Hence, in the present study we evaluated B deficiency and toxicity on *in vitro* growing 'Aggezi Shami' and 'Picual' olive cultivars as a first step toward improving our knowledge of *in vitro* olive culture.

Material and Methods

The current research was carried out during 2018 at the laboratory of Pomology Department, Faculty of Agriculture, Cairo University, Giza, Egypt.

Plant material and culture conditions

Active growing shoots were collected from mature olive (*Olea europea*) trees of 'Aggezi Shami' and 'Picual' cultivars. 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 and used as explants immediately.

Micropropagation

Olive nodal cuttings were cultured on Rugini Olive Media (Rugini, 1984), supplemented with zeatin at 2.5 $mg.L^{-1}$, 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 $\mu mol\ m^{-2}\ s^{-1}$ cool-white fluorescent lamps). After four weeks, the sprouted buds were transferred to fresh media with the same composition.

Boron treatments

Olive shoots of the 3rd subculture were used for B treatments, shoots were cultured on ROM medium, five treatments of B concentrations were included in the experiment (0, 12.6 (control), 50, 100 and 150 $mg.L^{-1}$ of boric acid) to culture medium. All cultures were maintained in growth chamber at 25°C and 16h., photoperiod. At the end of experiment (8 weeks) plantlets were removed from the culture media and gently washed with tap water.

Growth parameters

The following parameters were recorded; survival percentage, shoot number, shoot length, total leaves number and plant fresh weight (FW).

Leaf chlorophyll content

Chlorophyll a and b were determined spectrophotometrically using 80% acetone as a solvent according to Lichtenthaler and Wellburn, (1983).

Leaf proline content

Free proline content was extracted from 0.5 g of fresh tissues in 3% (w/v) aqueous sulphosalicylic acid and estimated by ninhydrin reagent and the absorbance of the fraction with toluene was read at 520 nm (Bates *et al*., 1973).

Leaf boron content

Before determining the content of B, all samples were dried at 70°C and ground to a fine powder then digested using nitric acid, where it is an acceptable matrix for consistent recovery of metals which are compatible with the analytical method (Rice *et al*. 2017). B content in olive leaves was tested with Synchronous Vertical Dual View (SVDV) on Agilent 5100 Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES; Nyomora *et al*. 1997). For each series of measurements intensity calibration curve was constructed composed of a blank and three or more standards from Merck company (Germany). Accuracy and precision of the element measurement was confirmed using Merck's external reference standards and standard reference material for trace elements in water and quality control sample from National Institute of Standards and Technology (NIST), were used to confirm the instrument reading.

Statistical analysis

The treatments were arranged in a complete randomized design, data were subjected to analysis of variance (ANOVA) using the SAS software (version 9.0; SAS Institute, Cary, NC). The mean and standard error (SE) were calculated from three replicates per treatment.

Table 1: The effect of B concentrations on survival (%), shoot number, leaf number, shoot length and shoot fresh weight.

Cultivars	Concentrations of B(mg L^{-1})	Survival	Shoot number	Shoot length	Leaf No.	Fresh weight
Aggezi Shami	0	88.00 b	1.330 c	5.167 b	9.333 c	0.146 a
	12.5	100.0 a	2.020 bc	6.500 ab	12.67 ab	0.183 a
	50	100.0 a	2.057 ab	7.500 a	13.33 a	0.179 a
	100	100.0 a	2.777 a	5.300 b	10.23 bc	0.186 a
	150	85.00 c	2.420 ab	1.633 c	4.667 d	0.126 a
	Mean	**95.2**	**2.121**	**5.253**	**10.047**	**0.165**
Picual	0	71.00 c	1.133 a	4.000 bc	7.833 b	0.160 a
	12.5	100.0 a	1.217 a	5.083 ab	10.60 a	0.233 a
	50	88.00 b	1.453 a	5.433 a	10.57 a	0.183 a
	100	77.00 c	1.440 a	3.333 c	8.233 b	0.126 a
	150	62.00 d	1.727 a	1.567 d	6.067 c	0.104 a
	Mean	**79.6**	**1.394**	**3.883**	**8.66**	**0.141**
Means with the same letter are not significantly different at p <0.01.						

The significance of the differences among control and B treatments for each cultivar was evaluated with Duncan range test at 1% level (Duncan, 1955).

Results and Discussion

Data in table 1, showed that survival percentage decreased significantly under both deficiency and high B concentration in culture media, high B concentration had significantly increased mortality in both cultivars. Obviously, the effect of B toxicity on 'Picual' cv. was more evident than 'Aggezi Shami' cv. The obtained results concerning the growth parameters under different B concentrations showed that B deficiency and toxicity affected all plant growth parameters (Fig. 1). The highest value for shoot length and number of leaves was recorded in control treatment (12.5 mg.L^{-1} of B) and low B concentration (50 mg.L^{-1} of B), while the high B concentration (150 mg.L^{-1}) recorded the lowest values. There was no significant different in the shoot Fresh weight. Previous studies also reported that the high concentrations and toxicity of boron can lead to significant symptoms such as leaves chlorosis and growth inhibition (Goldbach *et al*., 2001; Han *et al*. 2009; Herrera-Rodríguez *et al*., 2010; Reid and Fitzpatrick, 2009; Sheng *et al*., 2010). The effect of B on the shoot fresh weight may be attributed to the role of B in the regulation of water relations of plants (Bennett, 1993). Mouhtaridou *et al*., (2004) reported that the *in vitro* cultures of apple shoots produced the highest fresh mass in low B medium compared with higher B concentrations. Cell division may also be reduced by low B (Robertson and Loughman, 1974). The differences in genotype response to B were

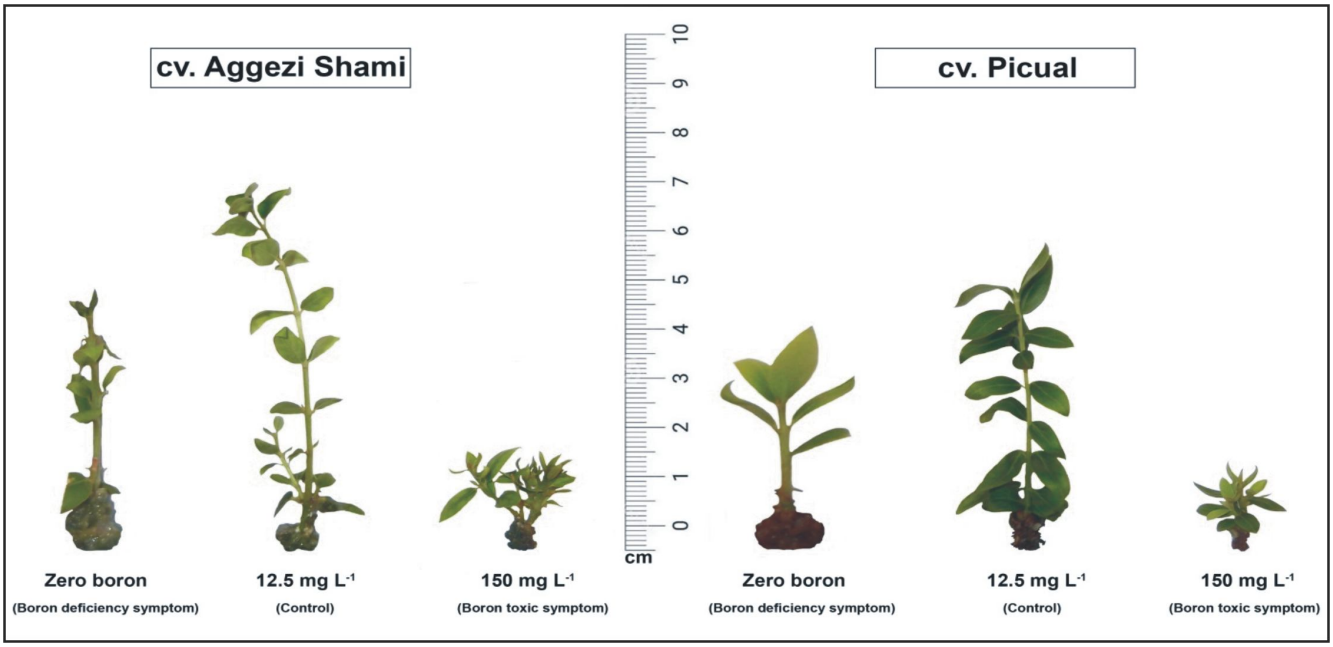

Fig. 1: Boron deficiency and toxicity symptoms on *in vitro* growing 'Aggezi Shami' and 'Picual' olive cvs. compared to control.

Table 2: The effect of B concentrations on leaf chlorophyll, proline and boron content.

Cultivars	Concentrations of B(mg L^{-1})	Ch a	Ch b	Proline	Boron
Aggezi Shami	0	0.035 b	0.098 d	0.0103 a	1.467 d
	12.5	0.421 a	1.591 a	0.0140 a	18.08 c
	50	0.164 b	0.612 b	0.0133 a	19.67 c
	100	0.092 b	0.227 c	0.0103 a	35.33 b
	150	0.024 b	0.057 d	0.0186 a	54.17 a
	Mean	**0.145**	**0.388**	**0.013**	**95.20**
Picual	0	0.014 b	0.050 b	0.0106 a	7.917 d
	12.5	0.482 a	1.391 a	0.0137 a	13.83 c
	50	0.132 b	0.365 b	0.0140 a	18.00 c
	100	0.051 b	0.160 b	0.0123 a	34.03 b
	150	0.038 b	0.116 b	0.0100 a	49.06 a
	Mean	**0.119**	**0.371**	**0.012**	**79.60**

Means with the same letter are not significantly different at p <0.01.

reported previously by Guidong *et al*., (2011) they found that plants grafted on citrange rootstock were more tolerant to B toxicity than the trifoliate orange-grafted plants. B uptake differs among plant species and cultivars. A high genotypic variation in B stress toleration ability of plants against excess B was reported in literatures (Hayes and Reid, 2004; Paull *et al*., 1992; Torun *et al*., 2006; Zhou *et al*., 2014). Sotiropoulos *et al*., (1998) pointed out that *"Actinidia arguta"* produced shorter and fewer shoots under *in vitro* B stress in comparison to *"A. deliciosa*", meaning that the genotype "*A. deliciosa*" may be able to maintain growth better than *"A. arguta"* at equivalent B concentrations. Similar observations are reported in different crops species including tomato, celery and wheat (Bellaloui and Brown, 1998), *Prunus* rootstocks (El-Motaium *et al*., 1994), kiwifruit (Sotiropoulos *et al*., 1999) and citrus (Papadakis *et al*. 2003).

On the contrary, data in table 1, showed that, shoot number markedly increased under high B concentration. The increase in number of shoots may results from death of apical meristems (Goldbach *et al*., 2001). According to Sotiropoulos and Dimassi, (2004), the increase in shoot number with high B concentration may attribute to that B promote axillary bud formation or induce bud burst and growth. Furthermore, it may indicate a possible relationship between high B and auxin levels.

As shown in table 2 chlorophyll a and b concentrations were gradually decreased with increasing B in the medium, moreover leaves chlorophyll content were dramatically decreased under B deficiency stress. Pervious studied showed that B deûciency results in leaf chlorosis and photosynthesis reduction (Dong *et al*., 2016; Han *et al*., 2009; Liu *et al*., 2014). Also, Han *et al*., (2009) found that Chl a and Chl b contents decreased signiûcantly under B toxicity in citrus. Similar results were reported in Arabidopsis (Chen *et al*., 2014). Furthermore, Papadakis *et al*. (2004) observed that B toxicity resulted in a decrease of the leaf chlorophyll concentration and size of mesophyll cell chloroplasts. Sotiropoulos *et al*., (2006b) indicated that, chlorophyll content of pear leaves declined as B concentration of the culture medium increased. This effect may be attributed to the decrease in Fe under higher B concentrations of the medium, hence reduce the formation of δ-aminolevulinic acid and protochlorophyllide, the precursors of chlorophyll biosynthesis (Montvedt *et al*., 1991). Also, B deficiency reduces chlorophyll contents of leaves, which in turn affect Hill reaction activity and photosynthetic rate (Sharma and Ramchandra, 1990). Proline was not markedly changed under B toxicity stress in leaf tissues under high B concentrations. As expected, B gradually increased in olive leaves with increasing B concentration in the culture medium, high B concentrations in culture media results in a very toxic effect on the growth of olive explants of both cultivars. Under high B concentrations, 'Aggezi Shami' cv. accumulates higher B concentration compared with 'Picual' cv. Boron-tolerant varieties are characterized by a decreased B concentration in their leaf tissues in comparison with non-tolerant varieties (Nable *et al*., 1990). Moreover, tolerance mechanism to B toxicity is primarily associated with reduction of B accumulation in plant tissues (Ardic *et al*., 2009; Cervilla *et al*., 2007). Our results showed that, 'Aggezi Shami' cv. accumulate higher B concentration even it show better resistance and higher values of growth parameter under B stress conditions which may be attribute to cellular regulation mechanism to protect plant cell from B toxicity. Reid and Fitzpatrick, (2009) suggested that protection of intra-cellular processes was provided by exclusion of excess B from cytosol into the apoplast in plant cells. Seresinhe and Oertli, (1991) found that with increasing B concentration in the culture medium, B is accumulated in leaf cell walls and may ûnally intrude into cytoplasm and thus disturb cytoplasmic metabolism, resulting in B toxicity and reduced growth of explants (Matoh, 1997). In citrus, Martínez-Cuenca *et al*., (2015) illustrated that the B-tolerant lines were associated with re-regulation of B transport as well as activation of the antioxidant system.

Conclusion

The response of *in vitro* olive culture to boron stress is elucidated in our study. Generally, the two studied olive cultivars revealed different growth behaviour in response to boron stress. High concentrations of B in the culture medium had a very toxic effect on the two olive cultivars being studied. The boron-free medium also showed an obvious reduction compared to control in all parameter values. 'Aggezi Shami' cv. was more stress tolerant than 'Picual' cv. to boron.

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