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A New Approach for Olive (Arbequina cv.) Micropropagation: Effect of Dikegulac, Light and Carbon Source

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Abstract
Micro-propagation of olive provides the possibility of producing clones of native plants with appropriate attributes and mass reproduction in short period of time, compared to conventional methods. Apical dominance in olive explants limits the growth of lateral branches *in vitro*. The effect of Woody Plant Medium (WPM), Olive Medium (OM) and Murashig and Skoog (MS) with carbohydrate source (mannitol and sucrose), was investigated for *in vitro* shoot proliferation of olive. Different concentrations of sodium dikegulac (0.0, 2.5, 5.0, 7.5 and 10 mg L^{-1}) along with 3 mg L^{-1} zeatin and 0.5 mg L^{-1} BAP and two light qualities (red and white) were used to overcome apical dominance. Effects of different IBA concentrations on the *in vitro* root induction were also investigated. More than 74% of explants rooted when IBA was used at 2 mg L^{-1}. OM was the most effective medium, resulting in better and morphologically superior microshoots. Mannitol showed a positive effect on shoot proliferation. Using sodium dikegulac at 5 mg L^{-1} under white light significantly stimulated axillary bud growth. In the corresponding concentrations of sodium dikegulac, red light had a significant effect on longitudinal growth compared to the white light.

Keywords: Sodium dikegulac, apical dominance, light qualities, Arbequina.

Introduction

Olive (*Olea europaea* L.) is one of the most ancient domesticated fruit trees of the Mediterranean area. Tissue culture methods are extensively used for rapid multiplication of many plant species. Olive plants can be propagated from cuttings or by grafting on the seedling rootstocks (Fabbri et al., 2004). Additionally, the development of tissue culture techniques for mass propagation of olive plants has also received a considerable attention. However, olive is characterized by strong *in vitro* apical dominance (Rugini and Panelli, 1993), with little formation of secondary axillary shoots, thus limiting the *in vitro* micropropagation potential. To increase the proliferation rate, researchers have proposed various methods in extensive studies including the impact of environmental factors, light quality and the use of chemical compounds. Studies on the effects of light quality on the growth of lateral buds or somatic embryogenesis are limited (Hunter and Burritt, 2004). On the recent years, LED lamps have been widely used to enhance the growth rate of seedlings *in vitro* and their effects have been investigated on chlorophyll, photosynthesis,

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and morphogenesis in various plants. Light is the source of energy and the main element in the growth and development of a plant. All physiological processes, from germination to fruit production are influenced by light. However, plant responses to the light depend on its quality, intensity and duration (Shahak et al., 2004; Jao et al., 2005). There are different light receptors in plants; each one having a particular function and are activated at different wavelengths. Light receptors that are effective in plant growth and development are UV-B receptors, red light receptors (phytochromes) and blue light receptors (cryptochromes) (Muleo and Thomas 1997). The concept of light quality refers to the light source with different colours and wavelengths which affect anatomy, leaf size, chlorophyll content, shoot length, axillary shooting and rooting (Muleo et al., 2001), differentiation, regeneration, and shoot induction processes (Rossi et al., 1993).

Considering the dwarfing effect of sodium dikegulac and the height elongation effect of the red light, the aim of simultaneous application of these two factors was to increase shooting rate without negative effect on the longitudinal growth. The stimulation of axillary buds and the subsequent shoot elongation requires the presence of high concentrations of zeatin, a costly cytokinin which contributes considerably to the high final cost of micropropagated plants. Sodium dikegulac reduces apical dominance and promotes lateral branching and flower-bud formation in some plants (Bocion et al., 1975; Norcini et al., 1994; Das et al., 2006; Gyres and Mira 2008). Commercial formulations of dikegulac have been used as a pinching agent in several species (Malek et al., 1992; Jacyna et al., 1994; Sansberro et al., 2006; Rezazadeh et al. 2015), and fruit abscission agent in citrus plants (Pozo et al., 2004). Dikegulac was also tested in field-grown adult olives for its capacity to increase fruit set as due to its effects on temporary plant growth reduction, and an increase in the number of short shoots (Nir et al., 1983; Rugini and Pannelli 1993). An important factor in all culture media is the carbon source. Carbohydrates such as sucrose, mannitol, fructose and sorbitol serve as the main energy sources in plants. Garcia et al. (2002) reported that in Manzinillo cultivar, mannitol significantly promoted growth when compared to the effects of sucrose on growth. This effect was due to increasing shoot length, pairs of new leaves and breaking apical dominance.

Materials and Methods

Plant Material

Plant material including 3-year-old olive (*Olea europaea* L.) cv. Arbequina were obtained from the Research Department of Agriculture Jihad Organisation (Qazvin, Iran) in the summer of 2011. Zeatin was used in a fix concentration of 3 mg L^{-1}, therefore, it was not considered as a variable in the analyses.

Explant Sterilization

Green and semi-hard wood branches with apical and lateral buds were divided into small pieces. After removing leaves, the surface disinfection was carried out by washing explants under the running tap water for two to three hours. The explants were immersed in 96 % (v/v) ethanol for 2-3 seconds, followed by three washes by sterile distilled water for 5 min, then immersion in 0.1 % (w/v) $HgCl_2$ with two drops of Tween 80 for 5 min. Finally, explants were washed three times by sterile distilled water for 5 min.

Culture Establishment

Experimental plant material included single node segments each with two opposite buds. All explants were collected and established *in vitro* on OM medium (Duchefa, NL), plus 3 mg L^{-1}zeatin (Duchefa), 0.5 mg L^{-1} BAP (Duchefa), 3.6% mannitol (Duchefa), and 0.62%

phyto agar (Duchefa). Medium pH was adjusted to 5.70 before adding phyto agar and the medium was autoclaved for 20 min at 121 °C. All cultures were kept in a growth chamber at 24 ± 1 °C under a 16 h photoperiod (2000 Lux light intensity), provided by cool white fluorescent lamps (measured by the MS6612T digital light metre, white and coloured, South Africa).

Culture Medium and Carbon Source

In vitro cultures of olive explants were initiated in three different culture media, without growth regulators, i.e. Olive Medium (OM) (Rugini, 1984), Woody Plant Medium (WPM) (Lloyd and McCown, 1981) and Murashige and Skoog Medium (MS) (Murashige and Skoog, 1962), which were all supplemented with 36 g L^{-1} mannitol (Zacchini et al., 2004) or 30 g L^{-1} sucrose as the carbon source. Media pH was adjusted to 5.75 before adding the gelling agent. The media were solidified with 6.2 g L^{-1} Phyto agar. Cultures were incubated at 24 ± 1°C with a 16 h photoperiod provided by cool white fluorescent lamps (2000 Lux).

Light and Soduim Dikegulac

Nodal segments with two leaves, were excised and placed into glass jars containing 30 ml of OM medium (3 mg L^{-1}zeatin riboside and 0.5 mg L^{-1} BAP), plus dikegulac sodium (Sigma- Aldrich) (0.0, 2.5, 5.0, 7.5 and 10 mg L^{-1}) in two light qualities; fluorescent (2000 Lux) provided by cool white fluorescent lamps and red light (2000 Lux, provided by LED lamps) both measured by the MS6612T digital light meter. Zeatin and sodium dikegulac were filter-sterilized and added to the medium after autoclaving. Shoot proliferation was evaluated after 40 days of culture, using the same conditions as used for the stock material. The number of nodes, shoots and the length of shoots were recorded at the end of the culture period. Five explants per jar and four replications per treatment were used. Experiments were repeated for three times.

In vitro Rooting and Acclimatization

In the rooting stage, elongated shoots were compared with the control shoots and used as the experimental material. To evaluate the rooting potential of these shoots, each shoot having two to four nodes was cultured in the OM medium supplemented with different concentrations of IBA (0, 1, 2, 3 mg L^{-1}), 3.6 % mannitol (Duchefa) and 0.62 % Phyto agar (Duchefa). The pH of the rooting medium was adjusted to 5.75 before autoclaving. The tubes were then placed in a growth room in the dark for 5 days, and then transferred to the same environmental conditions used for the proliferation phase (Rugini and Fedeli 1990). The percentage of rooted shoots, the number of roots/shoot and root length were recorded after 30 days. All *in vitro*-rooted plants were transferred into Jiffy-Pots filled with a pitmus–perlite 3:1 (v/v) substrate.

Experimental Design

The experiments were conducted under a completely randomized design with three replications and 5 explants per replication and the data were evaluated by ANOVA analysis using statistical software SPSS 16.00. Normality of data was checked and if needed, logarithmic conversion or second root was used. Differences within treatments were estimated using Duncan test. Records were collected from the characters such as the number of shoots, stem elongation, leaf number and fresh weight.

Results

The effect of culture medium and carbon source on shoot proliferation

Analysis of results showed that OM medium was superior in comparison with WPM and MS media (Fig. 1) by producing the maximum number of shoots per proliferated explant. The results indicated that OM medium containing mannitol as the carbon source produced the maximum

number of shoots per explant. OM medium produced a higher number of healthy shoots (Fig. 1). The highest shoot length (11.35 ± 0.48 mm) was observed in the OM medium containing mannitol, while shoots with the lowest length (3.05 ± 0.22) were observed in the MS medium containing sucrose (Table 1). The shoots developed in the OM, WPM and MS media were also morphologically different to each other (data not shown). The maximum number of nodes per shoot (9.60 ± 0.46) was observed in the OM medium with mannitol, while the lowest number of nodes (3.05 ± 0.15) was observed in the MS medium containing sucrose (Table 1; Fig. 2).

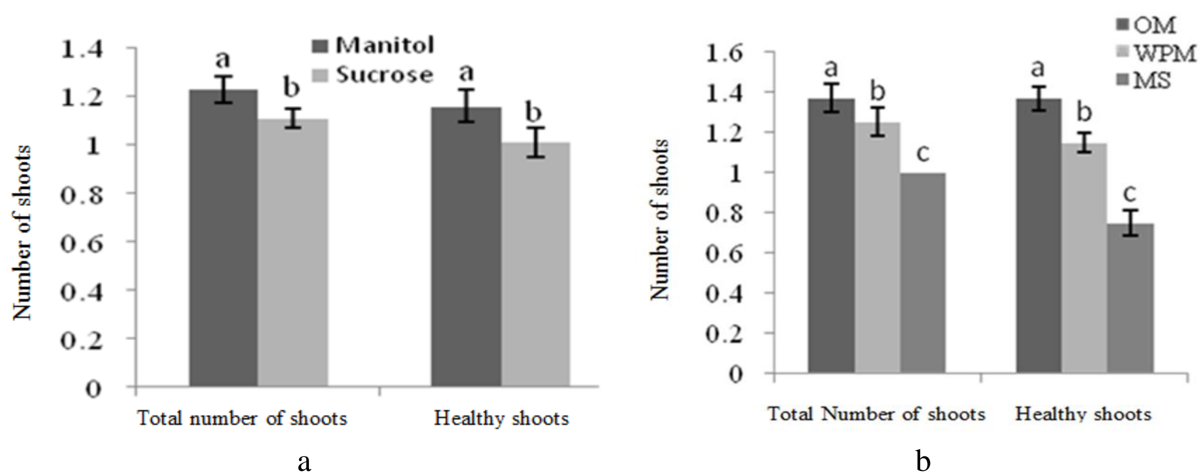

Fig. 1. Effects of different culture media and carbon sources on shoot number. a) carbon sources. b) culture media.

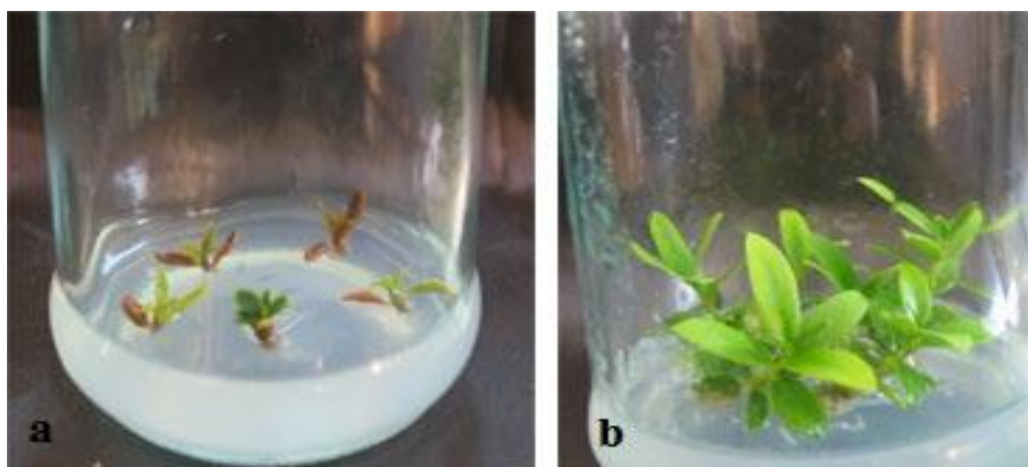

Fig. 2. *In vitro* shoot development. a) MS medium containing sucrose. b) OM medium containing mannitol.

Table 1. Effects of culture medium and carbon source on shoot length and nodes number.

Culture Medium	Carbon source	Shoot length (mm)	Nodes No.
OM	mannitol	11.35 ± 0.48	9.60 ± 0.46
	sucrose	7.10 ± 0.44	4.90 ± 0.35
WPM	mannitol	8.60 ± 0.49	5.75 ± 0.47
	sucrose	5.32 ± 0.30	5.40 ± 0.34
MS	mannitol	3.25 ± 0.23	4.0 ± 0.21
	sucrose	3.05 ± 0.22	3.05 ± 0.15

The effect of light and soduim dikegulac on shoot proliferation

Five different concentrations of sodium dikegulac in white and red lights were used. Effects of sodium dikegulac and the interaction between cultivar and sodium dikegulac were highly significant on all measured characteristics (Tables 2). White light had the highest effect on the number of shoots (2.9 ± 0.19) and leaf number (14.80 ± 0.47) at 5.0 mg L^{-1} sodium dikegulac. Although more than two shoots per explant were obtained at 7.5 mg L^{-1} sodium dikegulac, but a reduction in shoot length was observed. However, sodium dikegulac had a negative effect on the shoot length per explant (Table 3). The highest shoot length per explant (15.70 ± 0.51) was obtained when sodium dikegulac was not present in the culture medium (Table 2). In red light, the highest effect on the number of shoots (1.20 ± 0.09) was observed at 10 mg L^{-1} sodium dikegulac. However, sodium dikegulac had a negative effect on the shoot length and leaf number per explant (Table 2). The highest shoot length (19.75 ± 0.42) and leaf number per explant (11.15 ± 0.15) were obtained when sodium dikegulac was not present in the culture medium. Red and white lights increased longitudinal growth. The highest shoot and leaf number per explants were obtained at 5.0 mg L^{-1} sodium dikegulac under the white light without reducing shoot length. Red light had no effects on increasing the number of shoots, but it caused an increase in shoot length compared to the white light (Fig. 3). Also the highest shoot length was obtained under the red light.

In vitro rooting

The maximum rooting percentage (74.9 %) and number of roots (2.07 ± 0.37) were recorded at 2 mg L^{-1} IBA. The maximum root length (2.12 ± 0.04 cm) was recorded at 1 mg L^{-1} IBA (Fig. 4). Finally, rooted olive plants were transferred to greenhouse (Fig. 5).

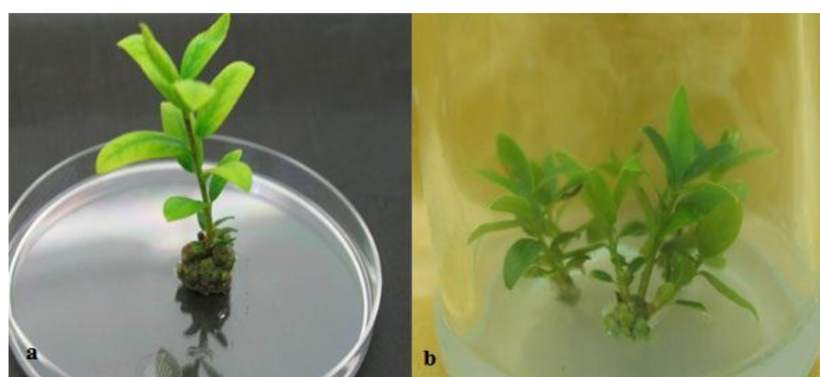

Fig. 3. Effect of sodium dikegulac under two different lights. A) *In vitro* shoot development in red light. B) *in vitro* shoot proliferation in white light.

Table 2. Effect of sodium dikegulac on shoot noumber, shoot length and leaf number (Means ± S.D.)

Light quality	Sodium dikegulac (mg L^{-1})	Shoot no. (per explant)	Shoot length (mm)	Leaf no. (per explant)
white	0.0	1.00 ± 0.00c	15.70 ± 0.51a	11.65 ± 0.44b
	2.5	1.15 ± 0.08c	13.95 ± 0.45b	11.40 ± 0.54b
	5.0	2.90 ± 0.19a	11.90 ± 0.25c	14.80 ± 0.47a
	7.5	2.15 ± 0.20b	9.55 ± 0.23d	7.50 ± 0.48c
	10	1.85 ± 0.16b	8.80 ± 0.30d	6.65 ± 0.42c
red	0.0	1.00 ± 0.00b	19.75 ± 0.42a	11.15 ± 0.15a
	2.5	1.00 ± 0.00b	18.00 ± 0.60b	9.95 ± 0.31ab
	5.0	1.00 ± 0.00b	14.05 ± 0.42c	8.35 ± 0.34b
	7.5	1.15 ± 0.08a	10.10 ± 0.37d	6.15 ± 0.34c
	10	1.20 ± 0.09a	10.20 ± 0.32d	6.10 ± 0.50c

Table 3. Effect of IBA on rooting (Means ± S.D.).

IBA (mg L^{-1})	Rooting (%)	Number of roots per shoot	Length of roots (cm)
1	57 ± 5a	1.75 ± 0.25ab	2.12 ± 0.04a
2	74 ± 9a	2.07 ± 0.37a	1.99 ± 0.28ab
3	35 ± 5b	1.25 ± 0.25b	1.49 ± 0.23b

Fig. 4. Root induction in 2 mg L^{-1} IBA.

Fig. 5. Rooted olive plant in the greenhouse after two weeks.

Discussion

Plants and tissues cultured *in vitro* require a carbon source since they are not fully autotrophic (Garcia et al., 2002). Generally speaking, sucrose is the carbohydrate of choice, probably because it is the main transport sugar in many plants. However, there are plant species that grow well on sugar alcohols such as sorbitol, glycerol and mannitol. In olive, mannitol is the major photosynthesis product (Flora and Madore 1998). For this reason, this polyalcohol could be a suitable choice for olive tissue culture (Garcia et al., 2002). Leva et al. (1994, 2013) reported that mannitol improved the *in vitro* propagation of olive. Mannitol is widely used in high concentrations to induce osmotic stresses. This polyalcohol is considered to be metabolically almost inert, therefore, mannitol-dependent phenomena are said to be osmotic stress (Steinitz, 1999). However, in species that photosynthetically produce mannitol and grow well on mannitol contained-*in vitro* conditions, the osmotic effect would be negligible. Why mannitol promotes the growth of olive explants *in vitro* more efficiently than sucrose is not known (Garcia et al., 2002). Pharr et al. (1995) proposed that the metabolic use of mannitol provides energetic advantages to plants, since the hexose-P generated from mannitol in sink cells is accompanied by the generation of two ATP molecules by conversion of mannitol. In contrast to mannitol, the initial generation of hexose-P from sucrose in sink cells occurs at the expense of ATP. The same could be true in olive metabolism (Garcia et al., 2002). The superiority of mannitol to sucrose in olive micropropagation was also evident in our study. In the initial experiments in the OM medium without sodium dikegulac, but including zeatin and BAP, there was no growth of lateral buds (data not shown). Zeatin is the only cytokinin that can enhance the growth of lateral buds and subsequent shoot elongation in olive micropropagation (Rugini and Baldoni 2004).

In the present study, combining sodium dikegulac and zeatin with white light caused an increase in the number of shoots in comparison to red light. In accordance with this finding, the dynamics of shoot branching in plum was shown to be

influenced by blue and red lights (Muleo et al., 2001). Blue light induced an increase in the number of formed axillary buds and a decrease in bud outgrowth, while red light decreased bud formation and reduced the strength of apical dominance, as a result increasing bud outgrowth. It seems that sodium dikegulac at low concentrations (2.5 and 5 mg L^{-1}) along with white light promoted zeatin to act, but at high concentrations (7.5 and 10 mg L^{-1}) it inhibited the zeatin effect. Red light increased shoot length but sodium dikegulac inhibited it. According to the Mendoza et al., (2007) findings the optimal concentration to reduce the apical dominance by sodium dikegulac is different in olive cultivars. It seems that the combination of sodium dikegulac and zeatin with white light increased tissue growth in comparison to the red light. There is a possibility that low concentrations of sodium dikegulac facilitated zeatin activity, while its high concentrations inhibited its activity. The current study was aimed to increase the shoot proliferation rate by sodium dikegulac. Results were in accordance with those of Mendoza et al., (2007). However, according to the response of cultivars, the optimal concentration may be different.

The effect of light quality on axillary buds and somatic embryogenesis has been previously studied *in vitro* (Hunter and Burritt 2004). In the few recent years, LED lamps have been extensively used to increase seedling growth *in vitro* and also to investigate their effect on chlorophyll synthesis, photosynthesis and morphogenesis (Hahn et al., 2000; Park et al., 2010). It has been reported that red light can cause an increase in vegetative growth (Shahak et al., 2004), shoot length, petiol length, leaf dry weight (Morgan and Smith 1979) root growth (Ross et al., 1993), organogenesis and the release of axillary buds from apical dominance (Hunter and Burritt 2004). Donini et al. (2008) used three olive cultivars namely Koroneiki Picual and Frantoio under 4 light qualities including white, red, blue and green. The highest percentage of viability was obtained in white light with Koroneiki cultivar, whereas viability percentage of Picual explants showed a significant difference in white and green lights. Our findings showed the importance of simultaneous application of dikegulac and white light to overcome apical dominance and to obtain a suitable rate of micropropagation in olive. It was shown that light quality, especially red light, has an important impact on shoot regeneration and propagation, in Arbequina cultivar. This effect could be increased by the addition of dikegulak. These results could be applied for other olive cultivars as well, however, by considering the optimization of conditions for each cultivar.

Acknowledgments

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