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In Vitro Micropropagation of Olive (*Olea europaea* L.) 'Mission' by Nodal Segments

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ABSTRACT

Olive (*Olea europaea* L.) cultivars are mainly propagated by hardwood cuttings under mist unit of greenhouses. Such techniques are time consuming, laborious and have limited efficiency. *In vitro* propagation methods may be a good alternative for propagation of olive cultivars. Current study was conducted to establish a successful and high efficient method for micropropagation of olive 'Mission' via nodal segments. Results of this study showed that production of phenolic compounds and necrosis of explants may be controlled by submersing the explants in a mixture of 100 mg L^{-1} citric acid and ascorbic acid for 30 minutes, after submersion in water for 2 hours. The best proliferation rate and growth obtained in the presence of benzyladenine and gibberellic acid at the rate of 2.1, 2.08, and 0.6 mg L^{-1}, respectively. Proliferated explants rooted on MS/2 supplemented with 4 mg L^{-1} indolbutyric acid. Rooted explants adapted to outdoor condition by placing them under ordinary mist/cooling unit system of a greenhouse. The method described in this study is suitable for bulk propagation of olive 'Mission' in a period of three to four months; however the applicability of the method should be evaluated for other olive cultivars.

Key Words: Browning, Micropropagation, Olive, Phenolic compounds, Proliferation, Rooting

INTRODUCTION

Olive (*Olea europaea* L.) originated in eastern basin of Mediterranean sea and cultivated from ancient times. Olives are salt and drought tolerant plants and may be grown on poor soils. Such characteristics have let olives to be separated all around the world and cultivated as an important crop, and as a suitable tree for afforestation.
Olives may be propagated by different methods such as seeds, stem cuttings, layering, and grafting. Rooting of the hardwood cuttings in mist unit of greenhouses is the most common method for olive propagation. This method is relatively simple and preserves the genetic material and characteristics of maternal plants. Propagated plants also get mature and start fruiting earlier than seedlings. However, the efficiency of these methods is low and they also need a highly controlled condition for long times.

In vitro propagation techniques have been established for fast cloning of many fruit trees in recent years (Troncoso et al. 1999). Successful micropropagation of some olive cultivars have been reported previously (Rugini and Lavee 1992, Dimassi 1999). However, the success of these methods for olives is dependant to the cultivar and genetic background of the plant (Rugini et al. 1999). Some cultivars do not response to *in vitro* conditions, their proliferation rate is slow (Bartolini et al. 1990), rooting of explants is also limited, and many plantlets die in the acclimatization stage (Rugini et al. 1999). The aim of this paper was to investigate an efficient method for micropropagation of olive 'Mission'. 'Mission' is one of the most popular cultivars of olive. 'Mission' is an American cultivar, which is tolerant to cold, salinity, and olive knot bacterial disease. 'Mission' trees are hard to root and their propagation efficiency is low (Sadeghi 2002).

MATERIALS AND METHODS

This study was conducted at the plant tissue culture laboratory of the Department of Horticultural Science, Shiraz University, Shiraz, Iran. Plant material was involved current season branches of 9-year old 'Mission' trees, which brought from Kazeroon Olive Research Center, Kazeroon, Iran. Shoots were defoliated and cut in 50 mm long segments, and washed for 30 min in running tap water. In order to control of phenolic compounds production and necrosis of explants, shoot segments were immersed in a 1:1 mixtures of ascorbic acid and citric acid (0, 50+50, and 100+100 mg L^{-1}), after 2 hours submersion in distilled water. Axillary buds, excised from nodal segments, and 30 mm long nodal segments were exposed to sterilization procedure by immersion in 70% ethanol for 1 min, washing in sterile distilled water, then immersed in 10% sodium hypochloride for 10 min and washing again three times in sterile distilled water. In order to control of internal infections, 4 mg l^{-1} nano-silver particles (L-2000, NANO CID®, Iran) added to the media (Rostami and Shahsavar 2009).

Cuttings placed in 50×90 mm glass jars containing 30 ml of MS/2 basal medium supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} agar. Effects of different concentrations of growth regulators 6-Benzyladenine (BA), and Gibberellic acid (GA_3) were evaluated on proliferation of the explants. BA (0, 0.7, 1.4, and 2.1 mg L^{-1}) added to the media prior to autoclaving.

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The pH of the proliferation media was adjusted to 5.7 ± 0.1 with HCl 0.1N or NaOH 0.1N before autoclaving at 121°C for 15 minutes. Different GA_3 concentrations namely 0, 1.04, and 2.08 mg L^{-1} infiltrated into the proliferation media directly after autoclaving. Two explants per jar were used and cultures were incubated under a 16:8 h photoperiod of cool-white light (1250 Lux). Observations were made five weeks after incubation and the percentage of sprouted axillary buds, shoot length, and leaf number were recorded.

To evaluate *in vitro* rooting of olive explants, proliferated microshoots were cultured on a MS/2 medium supplemented with 0, 2, 4, and 6 mg L^{-1} concentrations of Indol-3-Butyric Acid (IBA) for 1 month, under the same environmental condition as described for proliferation stage. Rooting percent, root number, root length, and callus formation were recorded at the end of this period.

The plantlets were transplanted to jiffy pots at the end of rooting stage. Pots were placed in a greenhouse under 16:8 photoperiod, at day/night temperature 25/20 ºC, and 95% relative humidity. Relative humidity reduced from 95 to 60, and 30% during 3 weeks to complete acclimatization of plantlets to outdoor condition.
The experimental design of the all stages was a Complete Randomized Design (CRD), with 10 vessels per treatment, and 2 explants per vessel. Means were analyzes with Duncan's Multiple Range test at $P \leq 0.05$ using SPSS (v 16.0) for Windows software.

RESULTS

Nano-silver was very effective in disinfection of explants and the infection percentage reduced less than 5 percents. Submersion of shoots in water and the mixture of ascorbic acid and citric acid, reduced formation of phenolic compounds and prevented necrosis of the explants significantly (Fig. 1). Increasing concentration of the mixture resulted in better result.

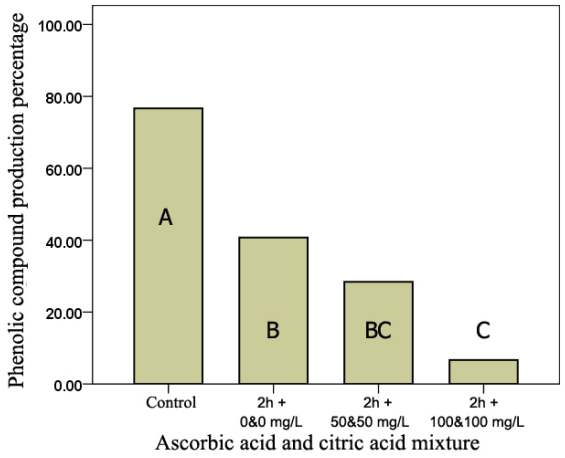

Figure 1. Effects of different concentration of ascorbic acid and citric acid mixture for 30 minutes, following 2 hours submersion in distilled water on accumulation of phenolic compounds in the proliferation media and necrosis of olive 'Mission' explants.
* Columns with the same letters did not show a significant difference in accordance to Duncan's Multiple Range Test, at $P \leq 0.05$.

Table 1 shows the effects of application of different concentrations of GA_3 and BA on proliferation of olive explants. Adding BA to the media, significantly increased the proliferation of the explants. Although GA_3 did not affect the proliferation of olive explants, the interaction of BA with GA_3 significantly increased proliferation rate of olive explants. The highest number of proliferated explants (100% explants per vessel) obtained in the presence of 2.08 and 2.10 mg L^{-1} of GA_3 and BA, respectively.

Table 1. Interaction of BA and GA_3 treatments in the presence of 0.6 mg L^{-1} NAA on proliferation (%) of olive 'Mission' explants.

	Mean Shoot Number							
	GA (mg L^{-1})						Mean	
BA (mg L^{-1})	0		1.04		2.08			
0	0.8†	cd	0.7	d	0.7	d	0.7	C
0.7	1.2	bcd	1.2	bcd	1.2	bcd	1.2	B
1.4	1.2	bcd	1.4	abc	1.6	ab	1.4	AB
2.1	1.3	bcd	1.4	abc	2	a	1.6	A
Mean	1.1	A	1.2	A	1.4	A		

†Means with the same letters did not show a significant difference in accordance to Duncan's Multiple Range Test, at $P \leq 0.05$.

Increasing BA and GA_3 concentrations in the proliferation media significantly increased length of olive shoots and the highest length of explants (12.5 mm) obtained in the presence of 2.08 and 2.10 mg L^{-1} of GA_3 and BA, respectively (Table 2). Increasing BA and GA_3 in the media significantly increased formation of leaves, and the maximum number of leaves (1.26 per explants) obtained in the media containing 2.08 and 2.10 mg L^{-1} of GA_3 and BA, respectively (Table 3).

Table 2. Interaction of BA and GA_3 treatments in the presence of 0.6 mg L^{-1} NAA on length (mm) of olive 'Mission' shoots.

	Mean Shoot Number							
	GA (mg L^{-1})						Mean	
BA (mg L^{-1})	0		1.04		2.08			
0	†21	e	47	b-e	68	bcd	45	B
0.7	37	cde	51	b-e	71	bcd	53	B
1.4	51	cde	57	cde	80	b	63	AB
2.1	35	de	77	bc	92	a	68	A
Mean	36	C	58	B	77	A		

† Means with the same letters did not show a significant difference in accordance to Duncan's Multiple Range Test, at $P \leq 0.05$.

Table 3. Interaction of BA and GA_3 treatments in the presence of 0.6 mg L^{-1} NAA on leaf number of olive 'Mission' explants.

	Mean Shoot Number							
	GA (mg L^{-1})						Mean	
BA (mg L^{-1})	0		1.04		2.08			
0	†0.00	c	0.00	c	0.26	bc	0.08	C
0.7	0.00	c	0.00	c	0.60	abc	0.20	BC
1.4	0.26	bc	0.53	abc	1.00	ab	0.60	AB
2.1	0.60	abc	0.86	abc	1.26	a	0.91	A
Mean	0.21	B	0.35	B	1.00	A		

† Means with the same letters did not show a significant difference in accordance to Duncan's Multiple Range Test, at $P \leq 0.05$.

Table 4 shows the results of the rooting experiment. The highest percentage of rooted explants (93.0%) obtained in the presence of 6 mg L^{-1} IBA in the rooting media and the lowest (0.0%) obtained in the control treatment. Number and length of roots significantly increased in response to increasing IBA concentration in the media up to 4 mg L^{-1} (Fig. 2). Callus formation increased following increasing IBA in the media. The highest percentage of callus (15.9%) observed in the presence of 6 mg L^{-1} IBA in the media. Although percentage of rooted explants increased following callus formation in the presence of high IBA concentration (6 mg L^{-1}), the length and the number of roots reduced under such condition.

Table 4. Root formation of olive explants to different concentration of IBA in MS/2 medium.

IBA Concentarion (mg L^{-1})	Callus formation (%)	Length of roots (cm)	Number of roots per explants	Rooting (%)
0	0.0 c	0.0 c	0.0 c	0.0 c†
2	8.0 b	1.6 ab	2.4 a	80.3 ab
4	10.3 ab	1.9 a	2.6 a	85.0 ab
6	15.9 a	1.4 ab	1.9 b	93.0 a

†Means with the same letters did not show a significant difference in accordance to Duncan's Multiple Range Test, at $P \leq 0.05$.

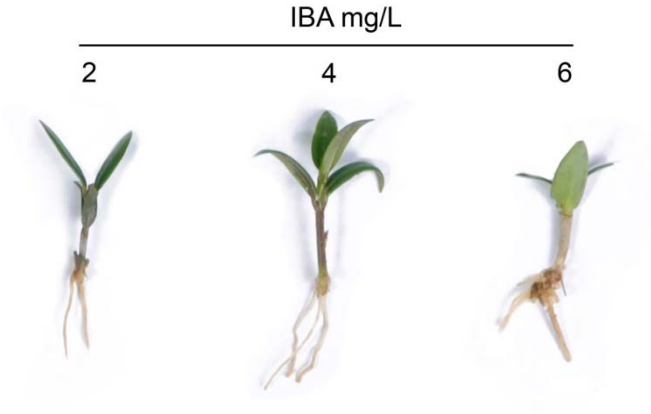

Figure 2. Increasing IBA concentration resulted in more callus formation, but reduction of growth of roots of olive 'Mission' explant.

Plantlets acclimated under mist unit in a greenhouse and transferred to outdoor after 3 weeks successfully. About 12% of the plantlets lost during this step.

DISCUSSION

Establishing a sterile *in vitro* culture is a challenging step in woody plant micropropagation. The step is time consuming and costly. In the current study approximately 95% of the node explants remained aseptic following adding 4 mg L^{-1} nano-silver particles to the proliferation media. As we described previously, using nano-silver particles in media is a safe and environmental friendly approach in controlling *in vitro* contaminations (Rostami and Shahsavar, 2009). Nano-silver particles release silver ions, which can break down the membrane structures of microorganisms (Dibrov et al. 2002).

Many plants produce dark phenolic substances after wounding. Accumulation of such compounds in medium adversely affects the growth and survival of *in vitro* explants. Roussos and Pontikis (2001) showed that accumulation of these compounds leads to necrosis and death of olive explants. Different methods such as adding antioxidants or activated charcoal to medium, keeping explants in dark; or submersing explants in water for periods of times have been reported to be effective in controlling phenolic compounds production. The success of these methods is dependant to species and\or cultivar. In the current study, submersion of explants in ascorbic acid and citric acid for 30 minutes as antioxidants, after 2 hours submersing in water, significantly reduced the production of phenolic compounds. Submersing the explants in water prevents phenolic compounds accumulation due to delusion of phenolic compounds and or their precursors in the explants (Rugini, 1984). Seyhan and Ozzambak (1994), and Roussos and Pontikis (2001) also showed that antioxidants reduce phenolic compound production and browning of olive explants.

Different hormonal mixtures are suggested for proliferation of olive cultivars. Our results showed that cytokinins are essential for *in vitro* proliferation of olive 'Mission' nodal explants. Although zeatin has been suggested for proliferation of olive explants (Chaari-Rkhis et al. 2003, Sghir et al. 2005), our results showed that BA also can be used in proliferation of olive explants. It should be noted that high cost of zeatin limits its usage in commercial micropropagation and there are many studies investigating on replacing zeatin with other types of cytokinins (García-Férriz et al. 2002). In most cases, using synthetic cytokinins such as kinetin and BAP in media did not show a proper performance in proliferation of olive explants. However, Dimmasi (1999) reported BA can be used in proliferation of olive 'Kalamon'.

Increasing BA concentration up to 2.1 mg L^{-1} in media increased proliferation rate of olive 'Mission' explants significantly (Table 1); however, there was some evidences of shoot growth limitation following increasing BA concentration in the proliferation media (Table 2). Debreg (1983) also has reported the side effects of BAP on growth of proliferated explants. He reported that abnormalities, especially vitrification, increase in the presence of high concentrations of BAP. Although increasing BA resulted in limited growth of explants, no signs of vitrification and other abnormalities did not observed. The lower BA concentrations did not affect the growth of the explants; however the rate of proliferation were lower in the lower concentration. Our results showed that the side effects of high concentrations of BA may be prevented by adding GA_3 to the proliferation media.

Adding GA_3 to media not only improved the growth of explants, but significantly increased number of nodes and leaves. Dimassi-Theriou (1994) and Grigoriadou et al. (2002) also suggested using GA_3 in addition to BA and/or zeatin in proliferation media of olive cultivars. Nowadays, it is accepted that GA_3 may improve *in vitro* growth of explants of many species (Vengadesan et al. 2003). Peixe et al. (2007) stated that longer internodes are more vigorous and they respond better to proliferation and/or rooting media.

Rooting of plant parts in the presence or absence of auxins is a genotype dependent factor. IBA has been suggested as the best plant growth regulator to induce rooting of olive explants (Grigoriadou et al. 2002). Our results also showed that presence of IBA in the rooting media is necessary for rooting of olive 'Mission' explants. The best results of rooting of the explants obtained following adding 4 mg L^{-1} IBA to the rooting media. Increasing IBA concentration up to 6 mg L^{-1} in the media resulted in maximum number of rooted explants, but on the other hand the growth of roots reduced and more callus production was induced (Fig. 2); these findings are in accordance to Zrÿd (1988). Shifting to more callus production following higher auxin concentrations may prevent or delay growth of roots (Peixe et al., 2007).

Plantlets acclimatized to outdoor condition in jiffy pots without sterilization in a mist unit in a glasshouse. The percentage of survival was 88. The results showed that olive explants may be acclimatized to outdoor without intensive care or treatments, only by step by step decreasing in relative humidity of environment. It has been shown that survival rates for different cultivars during acclimatization step is highly dependent to cultivar and may vary between about 90 to 60 %.

The method for micropropagation of olive 'Mission' reported here may be used for commercial mass propagation of this cultivar, in a period of 90 to 120 days. However, the applicability of this method should be evaluated for other cultivars.

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