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Effects of Culture Medium and Mother Plant Condition on Almond (*Prunus dulcis* Mill.) Shoot Proliferation from Nodal Segments

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ABSTRACT

In this research, the effects of season (spring and winter), culture medium and 6-benzyladenine (BA) on almond shoot proliferation were evaluated. Nodal segments with a single bud from 4 year-old grafts of two late-flowering almond cultivars, 'Shahrood 8' and 'Shahrood 10' were used as explants. Fungal and bacterial infections were completely eliminated using an oven infusion solution of fresh olive leaves. In both cultivars, BA at 1.5 and 2 mg/l resulted in the highest proliferation rate and Murashige and Skoog (MS) medium was superior to Woody Plant Medium (WPM) and modified Quoirin and Lepoivre (MQL) medium. In 'Shahrood 8', the highest shoot proliferation rate (5.83 shoots/explant) occurred in winter, using 2 mg/l BA, while in spring and with the same hormonal regulation, only 4.00 shoots/explant were produced. Conversely, in 'Shahrood 10', an average of 5.5 shoots/explant was produced at 1.5 mg/l BA, which was significantly higher than with 2 mg/l BA, and no significant differences were found when comparing winter with spring.

Keywords: explant, late-flowering, nodal segments, olive leaf infusion
Abbreviations: BA, 6-benzyladenine; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog; **PGR**, plant growth regulator; **QL**, Quoirin and Lepoivre; **WPM**, Woody Plant Medium

INTRODUCTION

As happens with many fruit species (Rugini 1983; Hortmann *et al.* 1997), traditional means of almond vegetative propagation such as cutting and layering are not applicable due to significant problems with *in vivo* rooting. Additionally, seed-derived plant propagation should be avoided due to heterozygocity and segregation of characters (Miguel *et al.* 1996).

For almond (*Prunus dulcis* Mill.), adventitious shoots have been obtained from leaf explants (Miguel *et al.* 1996; Ainsley *et al.* 2000), juvenile explants or endosperm (Mehra and Mehra 1974; Seirlis *et al.* 1979; Rugini 1983; Bimal and Jha 1985; Ainsley *et al.* 2001), immature seeds (Isikalan *et al.* 2008) and, to a limited degree, from adult almond explants (Tabachnik and Kester 1977; Gurel and Gulsen 1998; Lauri *et al.* 2001; Channuntapipat *et al.* 2003; Akbas *et al.* 2009).

A strong limiting factor in almond production worldwide is late spring frost that can cause severe damage and account for serious economic losses. Late-blooming cultivars (Kester and Asay 1975) are therefore highly desired by farmers. Thus, it is important to establish effective protocols for shoot regeneration from pre-existing buds of adult trees of these varieties, as explants for commercial propagation. 'Shahrood 8' and 'Shahrood 10' are two late-flowering cultivars that were selected and introduced by the Shahrood Agricultural Research Center and for which we aimed to develop efficient protocols for shoot proliferation from nodal explants. In this research we focused on the establishment of aseptic *in vitro* cultures, studied the effect of different culture media on the regeneration ability of the mother nodal explants, and also tested the explants' quality when collected in spring or in winter.

MATERIALS AND METHODS

'Shahrood 8' and 'Shahrood 10' almond cultivars grafted onto a bitter almond seedling rootstock were used in this study. Nodal stem segments containing a single axillary bud were collected from actively growing shoots of 4 year-old grafts in spring and used as explants. Because almond is a deciduous tree and its buds enter dormancy during winter time, to prepare the material to put *in vitro* during this season, a cold treatment had to be previously applied. For this, 20-25 cm long stems were taken from the trees, washed with detergent and tap water, wrapped in a wet newspaper and stored at 4°C for 400 h, based on results obtained when assessing bud break after 0, 100 and 400 h of cold treatment (unpublished results). After the cold treatment, the bottom part of cuttings (about 5 cm) was put in water, and the cuttings were maintained at room temperature (20°C) under a 16-h photoperiod with 35-40 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Mahtab Co. 40 watt). After one week the buds started to grow and the nodal segments of the new shoots were used as explants (**Fig. 1**).

Preliminary work showed that using 10-15% Golrang (commercial bleach containing 5.25% sodium hypochlorite) for 10-15 min was not sufficient to eliminate fungal and bacterial contaminations. Therefore we explored the possibility of using the natural antibacterial properties of some plants (Takahashi *et al.* 2004: Ashour *et al.* 2005: Shekafandeh and Khosh-Khui 2007). In this experiment, besides Golrang bleach, an infusion of olive leaves was used for decontamination. The infusion solutions tested were prepared as follows: (1) 50 g of fresh expanded leaves of olive 'Shengeh' (previously washed in tap water with detergent) were dropped in 1 liter of boiled water and steamed (placed over boiling water) for 2 h or, as an alternative, (2) stored at about 50°C (in the oven), for 24 h. So, the treatments were: Treatment 1 (T1; Control): Golrang 15% for 15 min; Treatment 2 (T2): immersion for 20 min in olive infusion (prepared for 2 h at 95°C) followed by 15% Golrang for 15 min; Treatment 3 (T3): immersion for 20 min

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Fig. 1 New shoots induced in winter from stem cutting of almond cv 'Shahrood 8'.

in olive infusion (prepared for 24 h at 50°C) followed by 15% Golrang for 15 min.

After disinfection and thorough washing, the nodal segments were then trimmed to 1-1.5 cm-long pieces and inoculated on MS medium. Three weeks later, the emerging shoots were transferred to MS (Murashige and Skoog 1962), WPM (Woody Plant Medium; Lloyd and McCown 1981) or MQL (Modified Quoirin and Lepoivre 1977, by omitting $FeSO_4 \cdot 7H_2O$ and $Na_2EDTA \cdot 2H_2O$) media containing 0.1 mg/l indole-3-butyric acid (IBA) and different concentrations of 6-benzyladenine (BA) (0, 0.5, 1.0, 1.5, 2.0 2.5 and 3 mg/l). After another 5 weeks data were recorded.

All culture media were supplemented with 3% sucrose (Merck, Darmstadt, Germany) and solidified with 0.8% agar (Merck). Media pH was adjusted to 5.7 ± 0.05 prior to autoclaving at 1.2 atm (17.6 psi), 121°C, for 20 min. All cultures were maintained at 25 ± 2°C, under a 16-h photoperiod with 35-40 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

In each experiment a completely randomized design was applied, with 4 replicates (4 vessels) and 3 explants per vessel. Data were analyzed using SPSS statistical software (SPSS Inc. Chicago. USA, version 15). The means were compared using Duncan's multiple range test at 5% of probability.

To root the developed shoots, 2-cm long cuttings were obtained and treated with 2 mg/l IBA dissolved in half strength MS medium (½ macro- and micro- MS nutrients) solidified with 0.7% agar (Merck KGa 54271 Darsmstadt, Germany), in the dark for 7 days, and then transferred to ½ MS without plant growth regulators (PGRs); these were maintained in the above mentioned temperature and photoperiod. After 4 weeks the percentage plant rooting was recorded. Subsequently, 3-4 cm rooted shoots were transferred to a mixture of sand: soil: perlite (1: 1: 1, v/v) and maintained at 25±2°C with a 16 h photoperiod of 35-40 µmol m^{-1} s^{-1} provided by cool white fluorescent lamps.

RESULTS

The treatments with the infusion of fresh olive leaves completely eliminated the fungal contamination visible in 'Shahrood 8' control experiments, although only T3 was effective to eliminate also the bacterial contamination (**Table 1**). Additionally, it was with disinfection T3 that the highest regeneration ability was observed (51%). T3 was therefore also applied to 'Shahrood 10', in which it also proved to be efficient.

Table 1 Effects of fresh olive leaf infusion on explant contamination control of the almond cv. 'Shahrood 8'.

Treatments	Fungal contamination%	Bacterial contamination%	Explant growth%
Treatment 1[a] (control)	50	100	0
Treatment 2[b]	0	20	17.22
Treatment 3[c]	0	0	51

[a] Golrang 15% for 15min
[b] 20 min olive infusion (prepared for 2 h at 95°C) + Golrang 15% for 15 min
[c] 20 min olive infusion (prepared for 24 h at 50°C) + Golrang 15% for 15 min

Table 2 Interactions between plant growth regulator (BA) and different culture media on shoot proliferation and shoot length from nodal segments of almond cv. 'Shahrood 8'[†].

PGR	Average shoot number/explant			Mean
BA mg /l	MS	WPM	MQL	
0.0	1.00 g	1.00 g	1.00 g	1.00 E
0.5	1.75 fg	1.66 fg	1.66 fg	1.67 D
1.0	2.33 ef	2.66 dc	2.58 de	2.53 C
1.5	4.67 a	3.91 abc	3.92 abc	4.18 A
2.0	4.00 ab	4.66 a	4.50 a	4.39 A
2.5	3.33 bcd	3.25 bcd	3.16 bcd	3.25 B
3.0	3.91 abc	3.33 bcd	3.25 bcd	3.20 B
Mean	2.98 A	2.93 A	2.87 A	
	Average shoot length/explant (mm)			
0.0	16.08 a	16.00 a	14.83 ab	15.67 A
0.5	12.41 abcd	12.41 abcd	11.75 bcde	12.19 B
1.0	13.25 abc	13.25 abc	13.25 abc	13.25 B
1.5	9.5b cdef	9.5b edef	9.50 bcdef	9.50 C
2.0	9.83 cdef	9.8b cdef	9.33 bedef	9.67 C
2.5	8.50 def	8.41 ef	8.33 ef	8.44 CD
3.0	7.33 f	6.92 f	6.92 f	7.06 D
Mean	11.00 A	10.92 A	10.56 A	

[†]Means with same letters (small letters for treatments and capital letters for main effects) are not significant at 5% level of probability using Duncan's multiple range test. PGR: BA= 6-benzyladenine, MS = Murashige and Skoog (1962), WPM=Woody Plant Medium - Lloyd and McCown (1981), MQL = Quoirin and Lepoivre (1977) medium lacking the original iron source (see Materials and Methods)

Upon culture on MS medium the buds of nodal segments of both cultivars enlarged and produced one shoot each. After 3 weeks, these shoots were transferred to different BA-supplemented media leading to the proliferation of new shoots after 10 days of culture.

In 'Shahrood 8', regardless of the basal culture media, 1.5 and 2 mg/l BA produced higher proliferation rates, 4.18 and 4.39 shoots/explant, respectively (**Table 2**). In this cultivar, the interaction between different culture media and BA concentrations showed the highest proliferation rate (4.67 shoots/explant) on MS with 1.5 mg/l BA (**Fig. 2A**). However, this was not significantly different from the number of shoots obtained on WPM and MQL basal media when supplemented with 2 mg/l BA (4.66 and 4.5 shoots/explant, respectively). In all media, the number of shoots more than 5 mm in length (those that were counted) decreased with increasing BA concentrations (from 2 to 2.5 mg/l). Also, the average shoot length decreased with increasing BA concentration (from 0 to 3 mg/l) (**Table 2**). Longest shoots were observed in the control without PGRs (15.67 mm).

In 'Shahrood 10', irrespective of the BA concentration, shoot proliferation from nodal segments showed that MS and WPM media produced more shoots/explant than MQL medium (**Table 3**, **Fig. 2B**). On modified QL medium, the leaves of both cultivars turned yellow and became necrotic, which may due to the omission of the iron source in the medium. Additionally, shoots induced on MQL medium (with either 1 or 2.5 mg/l BA) showed necrosis (**Fig. 3**). The results demonstrated that there was a strong interaction between culture media and different BA concentrations. MS and WPM media induced the highest average shoot number/

Fig. 2 Aspects of shoot proliferation from nodal segment of almond 'Shahrood 8' (**A**) and 'Shahrood 10' (**B**), in winter, after 5 weeks of culture in MS medium with 1.5 mg/l BA.

Table 3 Interactions between plant growth regulator (BA) and different culture media on shoot proliferation and shoot length from nodal segments of almond 'Shahrood 10'[†].

PGR	Average shoot number/explant			Mean
BA mg/l	MS	WPM	MQL	
0.0	1.00 f	1.00 f	0.75 f	0.91 E
0.5	1.67 ef	1.67 ef	1.33 f	1.56 D
1.0	2.67 cd	2.67 cd	2.41 de	2.58 C
1.5	4.75 a	4.58 a	2.41 de	3.92 AB
2.0	4.75 a	4.75 a	3.33 bc	4.27 A
2.5	3.92 ab	3.92 ab	4.08 ab	3.97 AB
3.0	3.58 bc	3.58 bc	3.58 bc	3.58 B
Mean	3.19 A	3.16 A	2.56 B	
	Average shoot length/explant (mm)			
0.0	15.92 a	15.08 ab	12.5 abc	14.50 A
0.5	12.42 abc	11.42 bcd	11.41 bcd	11.75 B
1.0	13.25 ab	12.25 abc	12.25 abc	12.58 AB
1.5	12.58 abc	12.16 abc	12.16 bc	12.31 B
2.0	8.58 cd	8.33 cd	8.33 cd	8.42 C
2.5	8.75 cd	8.58 cd	8.58 cd	8.63 C
3.0	7.42 d	7.33 d	7.33 d	7.36 C
Mean	11.27 A	10.73 A	10.36 A	

[†]Means with same letters (small letters for treatments and capital letters for main effects) are not significant at 5% level of probability using Duncan's multiple range test. PGR: BA= 6-benzyladenine, MS = Murashige and Skoog (1962), WPM=Woody Plant Medium - Lloyd and McCown (1981), MQL = Quoirin and Lepoivre (1977) medium lacking the original iron source (see Materials and Methods).

Table 4 Seasonal effect of different BA concentrations on almond 'Shahrood 8' shoot proliferation[†].

PGR	Average shoot number/explant		Mean
BA mg /l	Winter	Spring	
0.0	1.25 fg	1.00 g	1.00 E
0.5	1.92 ef	1.75 efg	1.67 D
1.0	3.17 d	2.33 e	2.71 C
1.5	5.33 ab	4.67 bc	5.13 A
2.0	5.83 a	4.00 cd	4.67 A
2.5	4.41 c	3.33 d	3.67 B
3.0	4.75 bc	3.91 cd	3.79 B
Mean	3.80 A	2.98 B	

[†]Means with same letters (small letters for treatments and capital letters for main effects) are not significant at 5% level of probability using Duncan's multiple range test.

explant (4.75 with 2 mg/l BA) as compared to MQL with either 1.5 or 2 mg/l BA. However, shoots elongated better at 1.5 mg/l BA than at 2 mg/l.

The physiological conditions of the mother plant are important determinants for the successful response of *in vitro* cultures. In 'Shahrood 8', we found that shoot proliferation in winter was more abundant (3.80 shoots/explant) than in spring (2.98 shoots/explant). At all BA concentrations, nodal segments originating in winter from cold-treated cuttings produced more shoots than those from spring (**Table 4**). The highest shoot proliferation rate (5.83 shoots/explant) occurred in 2 mg/l BA in winter (**Fig. 2A**) and was significantly higher than that observed in spring (4.00). In 'Shahrood 10', the highest average number of shoots produced was also significantly higher in winter

Fig. 3 Shoots developed from almond 'Shahrood 10' explants, showing necrosis after 15 days of culture on MQL medium supplemented with 1 mg/l BA (**A**) or 2.5 mg/l BA (**B**).

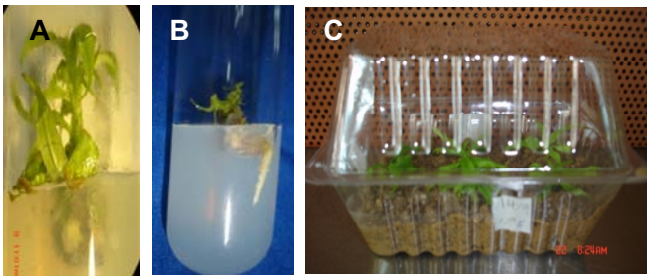

Fig. 4 ***In vitro*** **rooting of almond 'Shahrood 10'.** (**A**) Callus and root induction in 2 mg/l IBA. (**B**) Root elongation. (**C**) Establishment of rooted shoots in soil.

Table 5 Effect of different BA concentrations on winter *vs.* spring shoot proliferation from almond 'Shahrood 10'[†].

PGR	Average shoot number/explant		Mean
BA mg/l	Winter	Spring	
0.0	1.00 e	1.00 e	1.00 E
0.5	1.67 e	1.67 e	1.67 D
1.0	2.75 d	2.67 d	2.71 C
1.5	5.50 a	4.75 ab	5.13 A
2.0	4.58 b	4.75 ab	4.67 A
2.5	3.36 de	3.92 bc	3.67 B
3.0	4.00 bc	3.58 c	3.80 B
Mean	3.27 A	3.19 A	

[†]Means with same letters (small letters for treatments and capital letters for main effects) are not significant at 5% level of probability using Duncan's multiple range test.

(5.5) than in spring (4.75), but only when 1.5 mg/l BA was used (**Fig. 2B, 3**). At 2 mg/l BA the proliferation rates in winter and spring were not significantly different (4.58 and 4.75, respectively; **Table 5**). Moreover, about 50% of the shoots were capable of rooting in the culture medium after IBA treatment, and about 50% of them survived in soil mixture (**Fig. 4**). In preliminary experiments we also noticed that shoot induction media containing 0.5-1.5 mg/l BA produced shoots with higher rooting ability (over 50% of rooted shoots, with 2 roots/shoot) than with 2 mg/l BA (below 40% rooting and 1.8 roots/shoot).

DISCUSSION

Endogenous contamination of *in vitro* cultures of woody plants poses a serious problem (Pence and Sandoval 2005). The antimicrobial power of olive leaves against bacteria and fungi had been shown before by Markin *et al.* (2003) and Sudjana *et al.* (2009). Both teams investigated the effect of olive leaves extract on controlling microorganisms using agar dilution and broth micro-dilution technique. In our research, for the first time it was possible to reach a 100% success in eliminating fungal and bacterial contaminations of almond shoots, using an infusion of fresh olive leaves. This is a simple, safe and cheap method which can be used for *in vitro* explant decontamination.

Almond tissues have been grown in different basal culture media, depending on the goal of the work (Tabachnik and Kester 1977; Rugini 1983; Miguel *et al.* 1996; Chan-

nuntapipat *et al.* 2003). In this research, MS medium was more suitable than WPM or MQL media for shoot proliferation and growth which could be due to the higher concentration of total N in MS medium. Similar results were obtained from *in vitro* culture of wild cherry (Hammatt and Grant 1997). On modified QL medium, the leaves turned yellow and became necrotic which may have been due to the absence of iron sulfate and EDTA. However, QL medium has been reported as the best for shoot proliferation of the almond rootstock 'GF305' (Kalinina and Brown 2007) and MS medium has been mostly used for the *in vitro* establishment of almond 'Boa Casta' and 'Peneda' cultivars by Miguel *et al.* (1996) and Tereso *et al.* (2008).

Elevated BA concentrations were reported to increase shoot number/explant but decrease shoot length, and thus negatively affect shoot development (Tabachnik and Kester 1977; Rugini and Verma 1983; Kalinina and Brown 2007). This is similar to the results we obtained in our study (**Fig. 4B**).

The 400-h cold-treatment we applied to stem cuttings satisfied the chilling requirement of the buds. As a result, in 'Shahrood 8', winter nodal segments yielded more shoots than spring ones. The same effect, however, was not noticed for 'Shahrood 10', except when 1.5 mg/l BA was used. So, it is possible to micropropagate almond material throughout the year.

The media containing lower BA concentrations produced shoots with higher rooting ability, which is logic considering the negative effect of cytokinin on rooting. Our choice of applying darkness during the induction treatment was due to the fact that in first experiments it proved to be crucial to ensure rooting. Moreover, almond rooting was previously shown to improve when darkness is applied for the first week (Rugini *et al.* 1993; Tereso *et al*. 2008).

CONCLUSION

In the establishment of a micropropagation system for two late flowering almond cultivars released by the Shahrood Agricultural Research Center, we found that it is possible to use an infusion of fresh olive leaves that, together with a sodium hypochlorite treatment, ensures the complete decontamination of almond tissues, providing a cheap, rapid and non-toxic disinfection strategy. We also proved that winter nodal segments of the two cultivars may be efficiently used for micropropagation, provided the stem cuttings are previously subjected to a 400-h cold-treatment. This enlarges the period of plant material available for micropropagation.

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