

Effect of hot-water and cold treatments on reducing contamination in almond tissue culture

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Abstract

In this study, hot-water and cold treatments were used for eradication of explant contamination, and also the effect of plant growth regulators on shoot proliferation was evaluated. The explants were nodal segments of a late flowering almond cultivar 'Sharood 7'. Experiments were carried out in a complete randomized design with 25 replications. All hot-water treatments eliminated fungal contamination. The best hot-water treatment was 50°C in which 88% of explants were both free of contamination and necrosis followed by 76% at 47.5°C and 56% at 45°C. The best proliferation rate obtained in 1.5 mg L⁻¹ BA in combination with 0.1 mg L⁻¹ IBA (5.25 shoots per explant) which was significantly higher than 1 mg L⁻¹ (2.65 shoots per explant). Cold treatments only (2 and 4 days in 4°C) delayed fungal contaminations for 7 days, so it was impossible to assess bacterial contamination.

Key words: Benzyladenin (BA), late-flowering, nodal segments, proliferation, hot water

Introduction

In plant tissue culture, contamination (bacterial and fungal) is a major problem for both commercial and research laboratories (Cassells, 2001; Leifert and Waites, 1992). Plants that appear healthy may contain bacteria (Debergh and Vanderchaeghe, 1998; Legaatt *et al.*, 1988). These latent bacteria results in variable growth, reduced shoot proliferation, reduced rooting, tissue necrosis and finally cause death of tissue (Leifert *et al.*, 1989; Leifert and Waites, 1992). Using various combination of sterilants such as ethanol, hypochlorites, mercuric chloride, benolate and fungicides did not eliminate all contaminants (Kowalski and Staden, 1998). According to some studies, hot-water treatment (HWT) and cold treatment (CT) are very efficient in reducing initial contamination (Hol and Van Der Linde, 1992; Kowalski and Staden, 1998). In agricultural practices, HWT were used on a large scale with bulbs, tubers and seeds (Grondeau and Samson, 1994) and with grapevine cutting (Waite and Morton, 2007). The results from our preliminary work showed that explants contaminations were not completely controlled using sodium hypochlorite alone and with combination of benlate. In addition, mercuric chloride in concentration of 200 mg L⁻¹ removed fungal contamination but bacterial contamination appeared in second subculture after 8 weeks.

It has been indicated that the use of hot-water treatment in tissue culture is very efficient in reducing initial contamination of both bulbs (*Lilium*) and axillary buds of trees (*Acer*) (Langens-Gerrits *et al.*, 1998). Cold treatment of surface sterilized explant material of hardwood trees at 4°C for 2 or 4 days reduced or eradicated internal contamination (Kowalski and Staden, 1998).

Seasonal testing of plant material showed that contaminations were very low during winter months when night temperatures were around freezing (Kowalski and Staden, 1998). In the regions of cultivated almond in Fars province in Iran, late spring frost kills a lot of flower buds and even new branches each year.

Chilling injury can be avoided using late flowering cultivars, so these cultivars are in more demand. The aim of this research was decontamination of nodal segments of a late flowering local almond cultivar 'Sharood 7' for rapid clonal micropropagation.

Materials and methods

Three years old grafted almond (*Prunus dulcis* L.) trees cultivar 'Sharood 7' were grown in greenhouse condition. Nodal segments of 1.5-2.5 cm length from fresh shoots were used as explant. The collected nodal segments were washed in a commercial detergent (Rica) for 30 min, and then they were dipped in 70 % (v/v) ethanol, afterwards treated with 10% (v/v) Golrang solution, a house bleach (contain 5.25% sodium hypochlorite) and then rinsed three times with sterilized distilled water. A series of explants were maintained at 4°C for 0, 2 and 4 days. Another series was given hot-water treatments of 40, 42.5, 45, 47.5, 50 and 52.5°C in Ben-Mary for one hour. Treated explants were cultured on Murashige and Skoog (1962) medium supplemented with 3% sucrose (MERCK, LGaA64271 Darm stadt, Germany) and solidified by 0.8% agar (MERCK, LGaA64271 Darm stadt, Germany).

After 8 weeks, the explants were transferred to MS medium supplemented with different concentrations of BA (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg L⁻¹) and IBA (0.01 and 0.1 mg L⁻¹). The pH of all media were adjusted to 5.7 prior to autoclaving at 1.2 atm. pressure, 121°C for 15 min. All cultures were maintained at 25±2°C with a 16 h photoperiod of 35-40 μmol m⁻¹ s⁻¹ provided by cool white fluorescent lamps.

Each experiment was carried out as completely randomized design with 25 replications. Data were analyzed using SPSS (SPSS, Inc. Chicago, USA) and the means were compared using Tukey's test at *P*=0.05.

Results and discussion

Cold treatments (2 and 4 days at 4°C) delayed fungal contaminations for 7 days, thereafter fungi started to grow and covered the cultured vessels in few days, so it was not possible to evaluate bacterial contamination. In contrast, all hot-water treatments completely eliminated fungal contaminations. The results of hot-water treatments on bacterial contaminations, after 8 weeks of culture showed that with increasing water temperature from 40 to 52.5°C bacterial contamination rate decreased from 100 to 0%, respectively (Table 1).

Similar results were reported by Langens-Gerrits *et al.* (1998) on eradication of fungal and bacterial contamination in *in vitro* culture of *Lilium* bulb and *Acer* stem segments. Hot-water had a damaging effect on explant viability (Baker, 1962), so that, there was no explant alive after treating in 52.5°C hot-water. The best hot-water treatment was 50°C in which 88% of explants were both free of contamination and necrosis followed by 76% at 47.5°C and 56% at 45°C. During hot water treatment the explants were under partial anaerobic condition because of submerging which lead to production of acetaldehyde and ethanol (George, 1993). In *Prunus* accumulation of acetaldehyde and ethanol was followed by rapid deterioration (Righetti *et al.*, 1990). In *Malus* tissue culture, ethanol inhibited shoot proliferation and rooting (De Klerk *et al.*, 1997). After 8 weeks of explants establishments, they were subcultured on MS medium with different plant growth

Table 1. Effects of hot-water treatments on bacterial contamination of almond explants of cultivar 'Sharood 7'

Water temperature (°C)	Contaminated explants (%)	Decontaminated explants (%)	Explant survival (%)
40.0	100a [†]	0f	0f
42.5	96b	4e	4d
45.0	36c	64d	56c
47.5	24d	76c	76b
50.0	8e	92b	88a
52.5	0f	100a	0f

[†]Means in each column with similar letter(s) are not significantly different at $P=0.05$ using Tukey's test.

Table 2. Effect of plant growth regulators on shoot proliferation and shoot length in second subculture

Plant growth regulators		Average shoot number per explant	Average shoot length per explant (mm)
BA (mg L ⁻¹)	IBA (mg L ⁻¹)		
1	0.01	2.65c [†]	4.87b [†]
	0.10	3.48bc	5.21ab
1.5	0.01	4.63ab	6.47a
	0.10	5.25a	5.73ab
2	0.01	4.61ab	5.45ab
	0.10	3.40bc	4.74b
2.5	0.01	4.19ab	4.32b
	0.10	3.83bc	4.46b

[†]Means in each column with similar letter(s) are not significantly different at 5% level of probability using Tukey's test.

regulators. In this culture period (5 weeks), the explants remained free of contamination. The best proliferation rate obtained in 1.5 mg L⁻¹ BA in combination with 0.1 mg L⁻¹ IBA (5.25 shoots per explant) which was significantly higher than 1 (2.65 shoots per explant) and 2.5 mg L⁻¹ BA (3.83 shoots per explant) (Table 2).

In 2.5 mg L⁻¹ BA, the shoots showed deformation with yellow colour (Fig. 1). This may be due to competition among shoots in absorption of growth regulator and nutrient from the medium (Ali *et al.*, 2003).

Hot water (50°C) treatment was very effective in reducing exogenous and endogenous contaminations of almond cultivar 'Sharood 7'. This technique provides a cheap, rapid, non-toxic and efficient method for removing bacterial and fungal contaminations.

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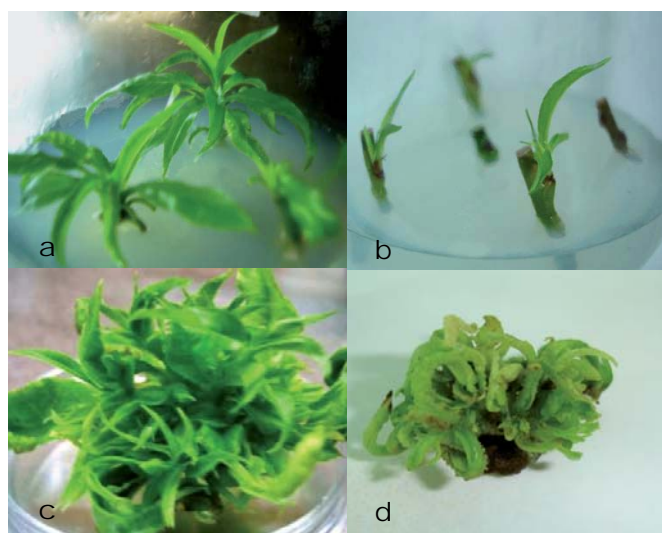


Fig. 1. Shoot proliferation in almond cultivar, 'Sharood 7'. a. bacterial contamination on the explants bases. b. contamination free explant and also necrosis explant c. shoot proliferation in 1.5 mg L⁻¹ BA. d. shoot deformation in 2.5 mg L⁻¹ BA.

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