Indian Journal of Experimental Biology
Vol. 53, July 2015, pp. 462-467

An efficient regeneration and rapid micropropagation protocol for Almond using dormant axillary buds as explants

Ravish Choudhary[1,2]*, Rekha Chaudhury[1], Surendra Kumar Malik[1] & Kailash Chandra Sharma[2]

[1]Tissue Culture and Cryopreservation Unit, National Bureau of Plant Genetic Resources (NBPGR), New Delhi-110 012, India
[2]Department of Botany, University of Rajasthan, Jaipur-302 055, Rajasthan, India

Received 09 January 2014; revised 29 May 2014

An efficient *in vitro* protocol was standardized for Almond (*Prunus dulcis*) propagation using dormant axillary buds as explants. Explants were cultured on Murashige and Skoog (MS) and woody plant medium (WPM) supplemented with different concentration/combination(s) of phytohormones. MS basal medium showed lowest shoot induction and took longest duration for shoot initiation. Multiple shoots were induced in MS medium supplemented with the combination of BAP (0.5 mgL^{-1}). Cultures showed poor response for rooting in all combinations of plant growth regulators (PGRs) and took 90 days for initiation. Rooting was higher in half strength of MS than in full-strength. The highest root induction (33.33%) was recorded in half MS medium supplemented with 0.1 mgL^{-1} IBA (indole-3-butyric acid) followed by full strength of MS medium (20%) supplemented with IBA (0.1 mgL^{-1}). α-Naphthalene acetic acid (NAA) was less effective for rooting than IBA. The highest root induction (25%) was found in half strength of MS medium supplemented with 0.1 mgL^{-1} NAA followed by full strength of MS medium (20%). The protocol developed would be of use in mass propagation of almond and also support *in vitro* conservation.

Keywords: Badam, Nuts, Propagation, *Prunus dulcis*, Rooting, Shoot induction, Sweet almond

Almond [*Prunus dulcis* (Miller) DA Webb., (Rosaceae)], is a large genus of deciduous or evergreen trees and shrubs, distributed chiefly in the temperate regions of the Northern hemisphere and is valued for its edible fruits/seeds and for ornamental flowers. The cultivated sweet almond has its origin traced to Central to South Western Asia[1]. It is one of the most important nut crops of temperate hilly regions of India, commercially grown in Jammu and Kashmir, Himachal Pradesh and Uttarakhand[2]. In the Himalayas, especially in the Khasi and Jaintia hills, almonds are being used locally as rootstocks for commercial stone fruits. Rich diversity of seedling populations is found in Kashmir (J & K) and Himachal Pradesh. Several cultivars developed in India and also those introduced as exotic collections such as Prianyi and Primorskij from Russia, White Brandis from Australia and others like Merced, Nonpareil, California, Paper Shell, Ne Plus Ultra, etc., now present good choice to farmers for improved cultivation. Almond, with its cultivated area of 21400 ha, registered productivity of 0.44 MT/ha with an annual production of 9340 MT[3].

*Correspondence:
Phone: +91 8750180494 (Mob)
E-mail: ravianu1110@gmail.com

Prunus seeds show poor germination due to low seed viability, seed dormancy and pathogens. Almond is highly heterozygous, and most common commercial cultivars are self-incompatible. Almond trees are virtually as variable as wild populations[4]. Vegetative propagation via layering or cutting has been inefficient in almonds. However, grafting and budding onto seedling rootstocks has been successfully used[5]. To maintain clonal purity, seed-derived material is not generally used for propagation. Thus, plant tissue culture techniques are more viable for clonal propagation of almond trees. Several attempts have been made to standardize *in vitro* regeneration protocols for almond cultivars and selections[4]. In almond, shoot regeneration is significantly dependent on the genotype, type of explants and combination of growth regulator used in the culture media[5]. Here, we have made an attempt to develop and standardize a reproducible *in vitro* propagation protocol of almond through dormant buds for quick regeneration.

Material and Methods

Winter dormant axillary buds of different almond cultivars (*Prunus dulcis*) were collected from the field genebank of Central Institute of Temperate

Horticulture (CITH), Srinagar, Jammu and Kashmir (J & K), during the winter period of January and February. *P. dulcis* during different seasons, its pre-mature fruits, mature fruits and twigs with dormant buds are shown in (Fig. 1).

Twigs (40 cm) were harvested from 1 year old lateral shoots of mature trees and ends of the twigs were immediately covered with wax at the ends of the twigs to maintain the moisture content (Fig. 1). Twigs were wrapped in cotton bags and kept in bucket of ice, and was air lifted to cryolab at NBPGR within 48 h of harvesting. In the laboratory, these germplasm were wrapped in polyethene bags and kept at −5 °C for experimentation within 25 days of harvesting.

Woody plant medium (WPM)[6] and Murashige and Skoog (MS)[7] medium were used with various concentrations and combinations of different plant growth regulators *viz.* BAP (0.1-1.0), TDZ (0.01-1.0), indole-3-acetic acid (IAA) (0.1-1.0), indole-3-butyric acid (IBA) (0.1-1.0) and α-naphthalene acetic acid (NAA) (0.1-1.0) @ mgL^{-1} were freshly prepared. All stock solutions were stored in glass reagent bottles (Borosil) and Fe-EDTA was stored in amber coloured glass bottle. All solutions were stored at 4 °C except nitrates, which was kept at room temperature (25 °C) to avoid crystallization.

All the media used in the present study were fortified with 3% sucrose and agar/phytagel. Different PGRs were used in combination based on the experiment designed. pH of the media was adjusted to 5.8 prior to autoclaving at 121 °C and 15 psi for 20 min. All the cultures were maintained at 25±2 °C under a 16 h light of 35 $\mu Em^{-2}s^{-1}$ (illuminated by cool white fluorescent tubes).

Culture initiation—The buds along with a small part of stem were carefully excised from twigs with the help of sharp surgical blade. 4-8 outer scales were removed using needles and blades fitted in scalpel without damaging the meristem. Buds enclosing meristems and has 6-7 scales (about 0.3-0.5 cm) treated with 1% (v/v) Tween 20 for 15 min. This was followed by washed under running tap water for 30 min to remove traces of the detergent. Eventually, the explants were aseptically surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 9 min, rinsed 4-5 times with sterile distilled water to remove the traces of sterilant with occasional gentle swirling. The surface sterilized meristems were inoculated on to semi-solid MS medium and WPM medium supplemented with varying concentrations and combinations of PGRs-BAP (0.1-2.0), TDZ (0.01-1.0), Gibberellic acid (GA_3) (0.1-0.5), Kn (0.1-1.0), Indole-3-acetic acid (0.1), IBA(0.01-1.0) and NAA (0.01-2.0).

Shoot induction—The explants were placed vertically in the culture tubes (25×150 mm) containing sterilized MS and WPM media supplemented with different combination of BAP (0.1, 0.5 and 1.0) and TDZ (0.01, 0.1 and 1.0) @ mgL^{-1} to optimize growth and differentiation. MS and WPM basal medium without any PGRs was used as a control. Bud sprouting was recorded fortnightly. The shoot induction percentage was recorded after 30 days of culturing.

Shoot elongation—For shoot elongation, sub-culture was done using nodal stem as a explant after 30 days of inoculation to the MS medium supplemented with either the same hormonal treatment used initially for shoot induction or separately in MS media supplemented with different combination of BAP (0.1, 0.5 and 1.0) and GA_3 (0.1, 0.2 and 0.5) @ mgL^{-1}. The shoot length was recorded after 40 days of sub-culturing.

Shoot multiplication—The elongated cultures were transferred on the optimal multiplication medium. The different combination and concentrations of BAP (0.1, 0.5 and 1.0), Kinetin (Kn) (0.1, 0.25, 0.5 and 1.0) and IAA (0.1) @ mgL^{-1} were used for shoot multiplication. Number of shoots per explants was recorded after 30 days of sub-culturing.

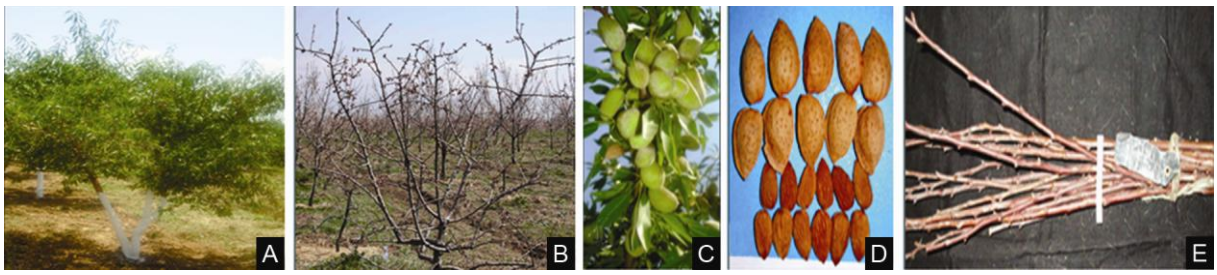

Fig. 1—*Prunus dulcis* in natural habitat (A) in summer; (B) in winter; (C) pre-mature fruits; (D) mature fruits; and (E) twigs with dormant buds.

Root induction—The cultures were transferred to half MS or full MS medium supplemented with various concentrations of auxins like IBA (0.1, 0.5 and 1.0) and NAA (0.1, 0.5 and 1.0) @ mgL^{-1}. The rooting parameters (number of roots produced by each explants and root length) were recorded after 20 days of initiation of roots. For each experiment, 25 explants per treatment were used and each experiment was repeated twice. The standard error (SE) of the arithmetic mean was calculated for individual treatment.

Results and Discussion

Shoot induction—The winter dormant buds of *P. dulcis* cultured on MS/WPM basal medium supplemented with different concentrations of BAP and TDZ, started sprouting after 17-45 days in most of the cultures. The response of explants for different combination of BAP and TDZ on shoot induction is presented in Table 1. In the case of WPM medium, maximum shoot induction (70%) and earlier bud sprouting (17 days) was observed with 1.0 mgL^{-1} BAP followed by 0.5 mgL^{-1} BAP (50%) and minimum (10%) was found with 1.0 mgL^{-1} TDZ (Fig. 2A & 2B, respectively). Using MS medium, highest shoot induction (60%) was also found in 1 mgL^{-1} BAP (Fig. 2D). Minimum shoot induction (30%) was observed using MS medium supplemented with 0.1 BAP and 0.01 TDZ @ mgL^{-1} (Fig. 2C). BAP showed higher shoot induction on WPM as well as MS medium.

In case of TDZ, maximum shoot induction (40%) was observed for dormant buds supplemented with MS+0.1 or 1.0 mgL^{-1} TDZ followed by dormant buds supplemented with WPM+0.1 mgL^{-1} TDZ (30%) (Table 1). Minimum shoot induction (10%) was observed in WPM medium supplemented with 1.0 mgL^{-1} TDZ. MS basal medium showed lowest shoot induction (10%) and took longest duration (45 days) for bud sprouting. Overall, the results demonsrated that WPM was the most suitable medium for shoot induction. The results also showed that the concentration of 1.0 mgL^{-1} BAP respond better for shoot induction and took less time for bud sprouting compared to other combination of BAP and TDZ. WPM medium was more effective compared to MS medium for shoot induction. Similarly, Yao *et al*.[8] have shown that WPM medium was significantly better than other media for regeneration of shoots from petioles of *P. domestica*, and in *P. serotina* by Espinosa *et al*.[9]. In cherry (*P. avium*), BAP was more effective as it led to more shoots[10]. On the other hand, TDZ has been shown to inhibit shoot elongation[11], shoot multiplication in *P. avium* cv. Lapins[10] and induce formation of shortened internodes in apple (*Malus domestica* cv. Gala)[12] in alignment with our results. Similar responses were also reported by a number of researchers with several woody species[13,14].

Shoot elongation—Following shoot induction (Fig. 3A), shoot elongation was recorded when the sprouted buds were sub-cultured in the same medium or MS medium supplemented with different combination of BAP and GA_3. However, no significant improvement was observed in any combination of PGRs. After 45 days of culturing on BAP medium elongation was noted (Fig. 3B). Sharifmoghaddam *et al*.[5] reported that BAP treatments led to better shoot proliferation and shoot multiplication.

Table 1—Shoot induction from dormant axillary bud explants of *Prunus dulcis* on MS and WPM medium supplemented with cytokinins

Concentration (mgL^{-1})	Average no of days*	Shoot induction (%)
MS Basal	45.00 (±2.72)	10.00 (±4.71)
MS + 0.01 TDZ	35.00 (±2.36)	30.00 (±4.71)
MS + 0.1 TDZ	21.00 (±0.47)	40.00 (±4.08)
MS + 1.0 TDZ	25.00 (±0.47)	40.00 (±4.08)
MS + 0.1 BAP	28.00 (±0.94)	30.00 (±4.71)
MS + 0.5 BAP	25.00 (±0.47)	40.00 (±4.08)
MS + 1.0 BAP	21.00 (±0.47)	60.00 (±4.71)
WPM Basal	40.00 (±4.08)	16.67 (±5.44)
WPM + 0.1 BAP	28.00 (±0.94)	30.00 (±4.71)
WPM + 0.5 BAP	25.00 (±0.47)	50.00 (±4.71)
WPM + 1.0 BAP	17.00 (±0.94)	70.00 (±2.36)
WPM + 0.01 TDZ	38.00 (±2.36)	20.00 (±4.71)
WPM + 0.1 TDZ	20.00 (±0.47)	30.00 (±4.71)
WPM + 1.0 TDZ	27.00 (±0.94)	10.00 (±4.71)

*Average no of days for bud sprouting

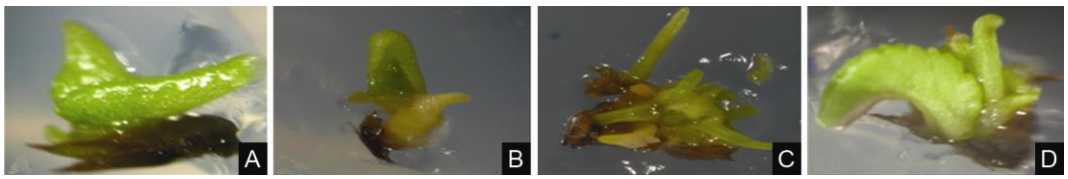

Fig 2—Effect of different medium on shoot induction of *Prunus dulcis*. (A) WPM+1.0 mgL^{-1} BAP; (B) WPM+0.5 mgL^{-1} BAP; (C) MS+0.1 mgL^{-1} TDZ; and (D) MS+1.0 mgL^{-1} BAP.

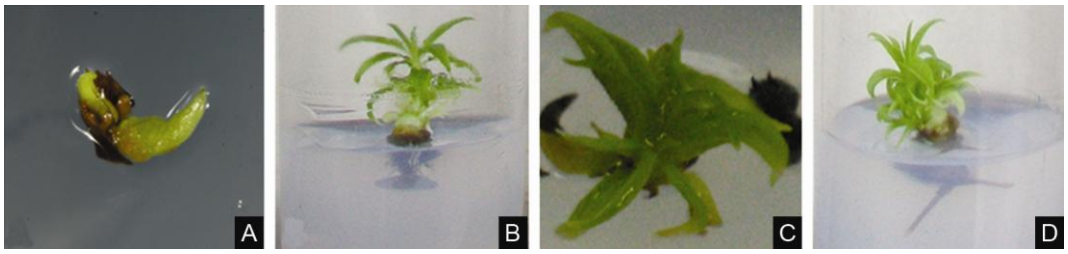

Fig. 3—*In vitro* regeneration of dormant buds of *Prunus Dulcis*: (A) shoot induction after 21 days; (B) elongation after 45 days; (C) shoot multiplication after 35 days; and (D) rooting after 90 days of culturing.

Table 2—Rooting of shoots regenerated from dormant axillary bud explants of *Prunus dulcis* on MS supplemented with different auxins and cytokinin combinations

BAP (mgL^{-1})	Auxin (mgL^{-1})	Effect of IBA for rooting (%)		Effect of NAA for rooting (%)	
		Half MS	Full MS	Half MS	Full MS
0.1	0.0	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
	0.1	33.33 (± 2.72)	20.00 (± 4.71)	25.00 (± 2.36)	16.67 (± 2.72)
	0.5	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
	1.0	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
0.5	0.0	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
	0.1	16.67 (± 2.72)	10.00 (± 4.71)	16.67 (± 2.72)	10.00 (± 4.71)
	0.5	6.67 (± 5.43)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
	1.0	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
1.0	0.0	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
	0.1	10.00 (± 4.71)	0.00 (± 0.00)	6.67 (± 5.43)	6.67 (± 5.43)
	0.5	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
	1.0	0.00 (± 0.00)	0.00 (± 0.00)	10.00 (± 4.71)	20.00 (± 4.71)

Shoot multiplication—No significant response was observed with Kn and IAA. Only 20% cultures showed multiple shoots (No of shoots 3-4) with MS + BAP (0.5 mgL^{-1}) after 30 days of transferring on fresh medium (Fig. 3C). No growth was observed when explants were cultured in the media without cytokinin or auxin[15]. Our results are in agreement with those of Tabachnick & Kester[16] and Isikalan *et al*.[17]. They reported that a cytokinin was necessary for development of *Amygdalus communis* L. Yapar *et al*.[18] reported poor response for new shoot multiplication.

Root induction—The percentage of root induction of *P. dulcis* is presented in Table 2. Cultures showed poor response for rooting in all combinations of PGRs. Root initiation was observed after 90 days of culturing. The highest root induction (33.33%) was recorded in half strength of MS medium supplemented with 0.1 mgL^{-1} IBA (Fig. 3D) followed by 20% rooting in full strength of MS medium supplemented with 0.1 mgL^{-1} IBA. Maximum number of roots/shoot (3.00) and highest root length (3.50 cm) was observed with half strength of MS medium supplemented with 0.1 BAP and 0.1 IBA @ mgL^{-1} (Table 3). NAA was less effective for rooting than IBA. Rooting success was higher in half strength of MS than in full-strength of MS. In the combination of NAA the highest root induction (25%) was found in half strength of MS medium supplemented with 0.1 mgL^{-1} NAA followed by 20% with full strength of MS medium supplemented with 0.1 mgL^{-1}. Maximum number of roots/shoot (2.5) and highest root length (2.80 cm) was observed with half strength of MS medium supplemented with 0.1BAP and 0.1 NAA @ mgL^{-1} (Table 3). Almond is particularly difficult to root both *in vivo* and *in vitro*[19]. Ainsley *et al*.[19] have shown that IBA and NAA are the most suitable auxin for rooting. No root development was observed in controls. However, IBA was effective for root induction; similar observation was found by Sharifmoghaddam *et al*.[5]. Channuntapipat *et al*.[20] showed that the maximum rooting of shoots for some almond hybrid rootstocks occurred on half strength MS medium with 2.4 mgL^{-1} IBA.

Table 3—Role of half and full strength of MS medium supplemented with different concentration and combination of IBA and NAA for number of roots and root length

Medium composition	IBA (0.1 mgL^{-1})		NAA (0.1 mgL^{-1})	
	No of roots/shoot	Root length (cm)	No of roots/shoot	Root length (cm)
½ MS+0.1 BAP	3.00 (±0.47)	3.50 (±0.24)	2.50 (±0.24)	2.80 (±0.09)
½ MS+0.5 BAP	2.00 (±0.47)	2.50 (±0.24)	2.00 (±0.47)	2.10 (±0.05)
½ MS +1.0 BAP	1.50 (±0.24)	1.60 (±0.25)	1.00 (±0.47)	1.30 (±0.14)
Full MS+0.1 BAP	2.00 (±0.47)	2.80 (±0.09)	2.00 (±0.47)	2.50 (±0.24)
Full MS+0.5 BAP	1.50 (±0.24)	2.20 (±0.16)	1.00 (±0.47)	1.90 (±0.05)
Full MS+1.0 BAP	1.00 (±0.47)	1.00 (±0.47)	1.00 (±0.47)	0.50 (±0.08)

Conclusion

WPM is a relatively low salt medium compared to MS medium. Almond needs a low concentration of salts for *in vitro* regeneration, and therefore, WPM medium was significantly better than MS medium for *in vitro* propagation. Obtained results clearly suggest that the cytokinins type and concentration suitable for micropropagation of woody plants are probably genotype-dependent, i.e., may depend on plant species. Thus, analysis of the effect of all cytokinins on regeneration phase of almond infers that BAP gives the best results. TDZ is used for micropropagation of wide array of woody species because of its ability to stimulate shoot proliferation. However, in this study, it did not stimulate shoot proliferation as compared to BAP. It is also concluded that proliferation of almond by tissue culture is a fast, economic and valuable method. Since in micropropagation rooting of plantlets is frequently a challenging step, losses at this stage have vast economic consequences. In conventional propagation via cuttings many woody plants are also recalcitrant to root. Thus, a research on root formation is highly important from the practical point of view. Rooting was higher in half strength of MS than in full-strength of MS. These results may serve as guidelines for improving propagation of almond tree. The protocol developed would be of great use for mass propagation of almond and also support for *in vitro* conservation.

Acknowledgement

Authors thank the Director, National Bureau of Plant Genetic Resources (NBPGR), New Delhi for the financial support.

References

1 Watkins R, Cherry, plum, peach, apricot and almond. *Prunus* spp. In: *Evolution of Crop Plants.* (Ed. Simmonds NW, Longman, London), 1976, 242.

2 Sharma SD, Kumar K, Gupta S, Rana JC, Sharma BD & Rathore DS, Temperate Fruits. In: *Plant Genetic Resources: Horticultural Crops*. (Eds. Dhillon BS, Tyagi RK, Saxena S & Randhawa GJ, Indian Society of Plant Genetic Resources, New Delhi, Narosa Publishing House Pvt. Ltd., New Delhi, India), 2005, 146.

3 Area and production of fruits and vegetables-All India (2012-13), In: *Indian Horticulture Database-2013*, (Ed. RK Tiwari; NC Mistry, B Singh & CP Gandhi, National Horticulture Board, Ministry of Agriculture, Govt. of India), 2014, 282. (http://www.nhb.gov.in/area-pro/Indian%20Horticulture% 202013.pdf. As accessed on 21 June 2015).

4 Sharifmoghaddam N, Safarnejad A & Tabatabaei SM, The effect of plant growth regulators on callus induction and regeneration of *Amygdalus communis*. *Not Sci Biol*, 3 (2011) 97.

5 Isikalan C, Namli S, Akbas F & Ak BE, Micrografting of almond (*Amygdalus communis*) cultivar 'Nonpareil'. *Aust J Crop Sci*, 5 (2011) 61.

6 Lloyd G & McCown B, Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. *Proc Int Plant Propagators' Soc*, 30 (1980) 421.

7 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol Plant*, 15 (1962) 473.

8 Yao YX, Sun YW, Li GG & Li GH, Regeneration of plants from *in vitro* culture of petioles in *Prunus domestica* lindl (European plum). *Biotech & Biotech*, 25 (2011) 2458.

9 Espinosa AC, Pijut PM & Michler CH, Adventurous shoot regeneration and rooting of *Prunus serotina in vitro* cultures. *Hort Sci*, 41 (2006) 193.

10 Rozic DV & Vujovic TI, The effects of cytokinin types and their concentration on *in vitro* multiplication of sweet cherry cv. Lapins (*Prunus avium* L.). *Hort Sci* (Prague) 35 (2008) 12.

11 Huetteman CA & Preece JE, Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tiss Org Cul*, 33 (1993) 105.

12 van Nieuwkerk JP, Zimmerman RH & Lordham, Thidiazuron stimulation of apple *in vitro*. *Hort Sci*, 21 (1986) 516.

13 Thengane SR, Kulkarni DK, Shrikhande VA, Joshi SP, Sonawane KB & Krishnamurthy KV, Influence of medium composition on callus induction and camptothecin(s) accumulation in *Nothapodytes foetida*. *Plant Cell Tiss Org Cul*, 72 (2003) 247.

14 Pruski K, Astatkie T & Nowak J, Tissue culture propagation of Mongolian cherry (*Prunus fruticosa*) and

Nanking cherry (*Prunus tomentosa*). *Plant Cell Tiss Org Cul*, 82 (2005) 207.

15 Akbas FA, Isikalan C, Namli S & Ak BE, Effect of plant growth regulators on *in vitro* shoot multiplication of *Amygdalus communis* L. cv. Yaltsinki. *Afr J Biotech*, 8 (2009) 6168.

16 Tabachnik L & Kester DE, Shoot culture for almond and almond-peach hybrid clones *in vitro*. *Hort Sci*, 12 (1977) 545.

17 Isikalan C, Akbas FA, Naml S, Tilkat E & Basaran D, *In vitro* micropropagation of almond *(Amygdalus communis* L. cv. Nonpareil). *Afr J Biotech*, 7 (2008) 1875.

18 Yapar A, Atay S, Kayacier A & Yetim H, Effects of different levels of salt and phosphate on some emulsion attributes of the common carp (*Cyprinus carpio* L., 1758). *Food Hydrocolloids*, 20 (2006) 825.

19 Ainsley PJ, Collins GG & Sedgley M, *In vitro* rooting of almond (*Prunus dulcis* Mill.). *In Vitro Cell Develop Biol Plant*, 37 (2001) 778.

20 Channuntapipat C, Sedgley M & Collins G, Micropropagation of almond cultivars Nonpareil and Ne Plus Ultra and the hybrid rootstock Titan. *Nemaguard Sci Hort*, 98 (2003) 473.