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MICROPROPAGATION STUDIES IN ALMOND (*Prunus dulcis* Mill.) cv. 'Shalimar'

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ABSTRACT

Surface-sterilized forced shoots tips were inoculated on different basal media supplemented with various combinations of growth regulators. Quality and quantity of growth regulators and nutrient media were found crucial for culture establishment. Optimum culture establishment was achieved on ½MS medium containing benzylamino purine (BAP) + indole butyric acid (IBA) [0.50 + 0.1 mg L^{-1}] where 63.33% morphogenetic response was observed. Callusing at the base of initiating cultures was minimum with BAP + IBA (0.25 + 0.01 mg L^{-1}). Micro-shoots from the established cultures were sub-cultured on MS media supplemented with BAP and NAA (naphthalene acetic acid) alone or in combination for axillary shoot proliferation. Maximum proliferated cultures (80%) with high number of shoots explant^{-1} (13.7) and proliferation grade (3.9) was obtained with BAP (0.40 mg L^{-1}) + NAA (0.01 mg L^{-1}). BAP proved superior to NAA during axillary shoot proliferation. Micro-shoots (10-15 mm) from proliferated cultures were subcultured in root induction medium (MS medium supplemented with IBA) and incubated under darkness for 10 days at 24±1°C and then transferred to root development medium (hormone-free MS medium) and incubated under normal culture room conditions. The rooting of micro-shoots, root length and number of roots shoot^{-1} were highest in treatment IBA (1.0 mg L^{-1}).

Key words: Almond, *in vitro* propagation, micro-propagation, proliferation, shoot-tip culture

INTRODUCTION

Modern biotechnological techniques have proved helpful in hastening the production of new genotypes and broadening the gene pool available for improvement of woody fruit species (Ainsley *et al*., 2001). However, *in vitro* culture and regeneration of adventitious shoots from adult fruit tree explants has proved a difficult task (Chanuntapipat, *et al.*, 2003). Conventional breeding of woody fruit species is a slow and difficult process due to high heterozygosity and long generation cycles (Sriskandarajah *et al*., 1994). Almond [*Prunus dulcis* (Miller) D.A. Webb.], belongs to the family Rosaceae and is native to the mountainous areas of Central Asia. In India, the major almond growing areas lie in Kashmir valley and Himachal Pradesh. In Jammu & Kashmir, the almond production is estimated to be 12,500 metric tonnes from an area of 17,650 ha (Anonymous, 2012). Major problems faced by the almond growers in India are low crop production, absence of defined varieties (as majority of almond plantations are of seedling origin), lack of quality planting material, etc. Due to the difficulties encountered in rooting of cuttings of almond cultivars, grafting and budding onto seedling rootstocks is the usual method used for vegetative propagation. These traditional propagation methods are time-consuming and have proved inadequate to meet the increasing demand of planting material of desired genotypes.

Micropropagation is the only viable alternative technique for increasing the supply of quality planting material. Tissue culture studies of almond have received less attention as compared to other stone fruits. Although some work has been conducted on almond (Tabachnik and Kester, 1977; Rugini and Verma, 1983; Kester *et al.,* 1986; Qadiri *et al.,* 2001) but no such studies have been carried out on the well-defined almond varieties especially in the Jammu and Kashmir State. In view of the urgent need for developing rapid multiplication techniques for almond, the present study was undertaken to standardize *in vitro* propagation protocol of almond cv. 'Shalimar' - a commercial cultivar released by SKUAST-Kashmir and well adapted to the agro-climatic conditions of J&K State.

MATERIALS AND METHODS

The present studies were carried out in Biotechnology Laboratory of the Division of Fruit Science, SKUAST-Kashmir, Shalimar, Srinagar (J&K) in the years 2006-2008. Forced almond explants were obtained from dormant hardwood cuttings (15-20 cm long and 10-15 mm in diameter) collected from the field-grown 25 years old mother plants of almond (*Prunus dulcis* Mill.) cv. 'Shalimar'. The cuttings were given 10 min. dip in 0.2% (w/v) fungicide captan 50 WP solution, dried under shade for 24 h and stored in a cold store in air tight poly-bags until use. Following one month of cold treatment, the basal ends of cuttings were re-cut by about 1 cm and placed in glass jars containing distilled water. These jars were then kept in incubation room maintained at 24±1°C with a 16 h photoperiod till sprouting of buds. The sprouting occurred 15-20 days after the transfer of jars to incubation room. The shoots putforth by sprouted buds on cuttings served as forced explant. The advantage of using forced shoot tips is that the explants are not only available in the off-season but are also free from the outside inoculum load (Dalal *et al.,* 2000).

Forced shoot tip explants were surface sterilized with mercuric chloride 0.1% (w/v) for 10 min. followed by rinsing the explants 4-5 times with double distilled sterile water to remove the traces of sterilants. Explants were prepared under laminar hood and inoculated on different media supplemented with various concentrations of benzyl amino-purine (BAP) or in combination with indole butyric acid (IBA) for the establishment of culture. Four media *viz.*, Murashige and Skoog's (1962) half strength medium [M_0], Murashige and Skoog's (1962) full strength medium [M_1], Anderson's (1978) medium [M_2] and Tabachnik and Kester (1977) medium [M_3] were used in the present investigation. The pH of the medium was adjusted to 5.7 with 0.1 N HCl/NaOH prior to the addition of agar. The media were autoclaved (1.06 kg cm^{-2} and 121°C) for 15 min. The cultures were maintained at 24±1°C under 16/8 h (D/L) photoperiod with 40±3 mol m^{-2} s^{-1} light intensity using 40W Phillips fluorescent tubes. Observations on the established and callused cultures were recorded within 5±1 week inoculation in establishment media.

The established explants were sub-cultured in proliferation medium within 5±1 week of culture initiation. The Murashige and Skoog's (1962) medium supplemented with BAP (0.00, 0.20, 0.40 and 0.60 mg L^{-1}) and NAA [naphthalene acetic acid] (0.00, 0.01, 0.02 and 0.03 mg L^{-1}) was used as basal medium during proliferation studies. Proliferation parameters were recorded within 5±1 week of culture in proliferation medium.

For *in vitro* rooting, well developed micro-shoots (10-15 mm) from the proliferating cultures were aseptically excised and cultured on root-initiation MS medium supplemented with different concentrations of IBA (0.5, 1.0 and 1.5 mg L^{-1}). Cultures were kept in darkness for 10 days in the culture room. Explants were then transferred to root development medium (hormone-free MS medium) and incubated under normal culture room conditions. Observations on the number of rooted micro-shoots, number of roots/shoot and maximum root length were recorded 4±1 weeks after inoculation in rooting medium. For each treatment 20 explants was cultured and all the experiments were repeated thrice. Data was analyzed using Minitab-12 statistical package and means were compared with LSD test.

RESULTS AND DISCUSSION

Culture establishment media were used for conditioning the explants and stimulating their initial growth (Fig. 1a). The first step of initiating *in vitro* culture is to successfully adapt the plant tissue or explant to heterotrophic mode of nutrition i.e. establishment stage. Effect of media on establishment of forced explants of almond was significant (Table 1). Maximum explant establishment of 37.22% was obtained when the explants were cultured in MS half strength medium followed by 34.16% in Anderson's medium and minimum of 26.38% with Tabachnik and Kester medium. Variations in response of explants to different types of media in terms of establishment may be due to the reason that high salt concentration of these media did not suit to explants during their initial *in vitro* growth in comparison to ½ MS medium which performed well due to its low salt concentration. These results are in close conformity with the findings of many researchers who have reported varied response of almond explants to different media during *in vitro* cultures (Bouza, 1997; Ainsley *et al.*, 2001; Qadiri *et al.*, 2002).

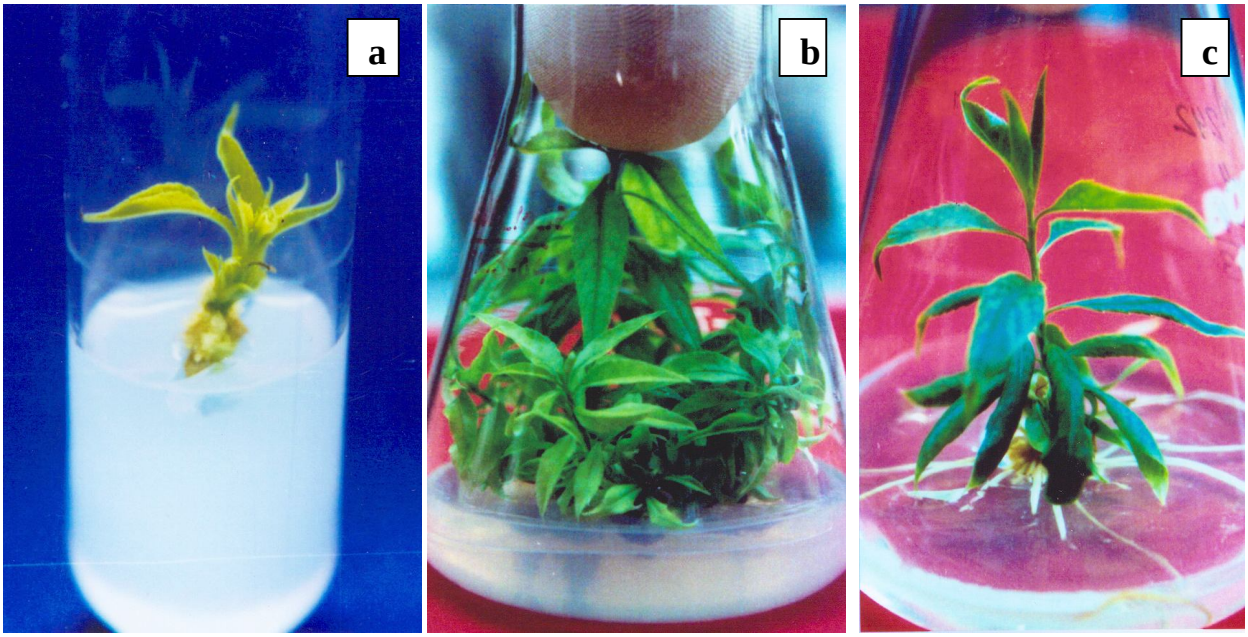

Fig. 1: Micropropagation of almond a) Explant establishment; b) Explant proliferation; c) Rooting

Table 1: Influence of nutrient media and growth regulators on *in vitro* culture of forced shoot tips of almond cv. 'Shalimar'

Growth regulators (mg L^{-1})	Per cent response to different media				Mean
	M_0	M_1	M_2	M_3	
BAP					
0.25	23.33 (28.85)	18.33 (25.30)	20.00 (26.56)	16.66 (24.04)	19.58 (26.18)
0.50	50.00 (45.00)	43.33 (41.16)	46.66 (43.18)	38.33 (38.24)	44.58 (41.89)
0.75	20.00 (26.56)	16.66 (24.04)	18.33 (25.30)	15.00 (22.79)	17.49 (24.67)
BAP 0.25 + IBA 0.01	41.66 (40.19)	33.33 (35.25)	38.33 (38.24)	30.00 (33.21)	35.83 (36.72)
BAP 0.50 + IBA 0.01	63.33 (52.74)	48.33 (44.04)	58.33 (49.80)	40.00 (39.23)	52.49 (46.45)
BAP 0.75 + IBA 0.01	25.00 (30.00)	20.00 (26.56)	23.33 (28.85)	18.33 (25.30)	21.66 (27.67)
Mean	37.22 (37.22)	29.99 (32.72)	34.16 (35.32)	26.38 (30.46)	
$CD_{0.05}$	Growth regulators: 1.26; Media: 1.03; Growth regulator x media interaction: 2.53				

Data in the parenthesis are arc sine transformed values of original percentages.

M_0 = Murashige and Skoog's half strength medium; M_1 = Murashige and Skoog's full strength medium; M_2 = Anderson's medium; M_3 = Tabachnik and Kester medium

Main effect of growth regulators revealed that BAP alone or in combination with IBA improved explant establishment. Optimum concentration of BAP was 0.5 mg L^{-1} which yielded an explant establishment of 44.58%. BAP concentrations below or above the 0.5 mg L^{-1} significantly reduced the explant establishment. Supplementation of BAP-fortified media with IBA further improved explant establishment. Highest explant establishment of 52.49% was observed when the explants were cultured in medium containing BAP + IBA (0.50 + 0.01 mg L^{-1}). Interaction between growth regulators and media was significant and highest explant establishment (63.33%) was recorded in ½ MS medium containing BAP + IBA (0.50 + 0.01 mg L^{-1}). Requirement of growth hormones is specific and it varies due to the endogenous levels of hormones in different tissues. Our findings are in line with Rugini and Verma (1983) who observed that shoot tips of almond cv. 'Ferragnes' established best on MS medium supplemented with 0.50 mg L^{-1} BAP and 0.01 mg L^{-1} IBA. Similar findings were also reported by Ishida *et al.* (1989) in *Prunus japonica* and by Shibli *et al.* (1996) and Ainsley *et al.* (2001) in almond and Aghaye (2012) in almond rootstock.

Main effect of basal media on callusing at the base of initiating cultures was significant (Table 2). Highest callusing at the base of cultures (60.27%) was observed when the explants were cultured on Murashige and Skoog's full strength medium and lowest (52.49%) when cultured on Murashige and Skoog's half strength medium.

Table 2: Influence of nutrient media and growth regulators on callusing of initiating cultures in forced shoot tips of almond cv. 'Shalimar'

Growth regulators (mg L^{-1})	Per cent response to different media				Mean
	M_0	M_1	M_2	M_3	
BAP					
0.25	41.66 (40.19)	48.33 (44.04)	43.33 (41.16)	45.00 (42.13)	44.58 (41.80)
0.50	58.33 (49.80)	66.66 (54.75)	61.66 (51.75)	63.33 (52.74)	62.49 (52.26)
0.75	81.66 (64.69)	93.33 (75.24)	86.66 (68.66)	90.00 (71.56)	87.91 (70.03)
BAP (0.25) + IBA (0.01)	25.00 (30.00)	31.66 (34.23)	26.66 (31.07)	28.33 (32.14)	27.91 (31.86)
BAP (0.50) + IBA (0.01)	43.33 (41.16)	50.00 (45.00)	46.66 (43.08)	48.33 (44.04)	47.08 (43.32)
BAP (0.75) + IBA (0.01)	65.00 (53.73)	71.66 (57.86)	66.66 (54.75)	68.33 (55.77)	67.91 (55.52)
Mean	52.49 (46.59)	60.27 (51.85)	55.27 (48.41)	57.22 (49.73)	-
$CD_{0.05}$	Growth regulators: 1.39; Media: 1.13; Growth regulator x media interaction : ns				

Data in the parenthesis are arc sine transformed values of original percentages.
M_0 = Murashige and Skoog's half strength medium; M_1 = Murashige and Skoog's full strength medium ; M_2 = Anderson's medium; M_3 = Tabachnik and Kester medium

Callusing of explants during establishment or proliferation stages is considered a negative factor and hence different growth regulator combinations/concentrations were used to minimize the phenomenon. BAP significantly influenced explant callusing. Minimum callusing at the base of cultures (44.58%) was observed when explants were cultured on medium supplemented with lowest concentration of BAP (0.25 mg L^{-1}) but increased with the increase in concentration of cytokinin with maximum (87.91%) in BAP [0.75 mg L^{-1}]. Incorporation of IBA in BAP supplemented media had a beneficial impact in reducing the callusing of cultures which was minimum (27.9%) in medium supplemented with BAP + IBA (0.25 + 0.01 mg L^{-1}). Our findings are in close conformity with Gurel and Gulsen (1998) who observed that benzyl adenine (BA) was essential for shoot development but higher levels caused callus formation which subsequently reduced the viability of shoots. Callus formation at the base of the shoots was also observed with BA or kinetin when used at a higher concentration in cherry cv. 'Maxma-14', a semi-dwarfing rootstock (Muna *et al.*, 1999).

Proliferation through stimulation of axillary buds is the most suitable method for multiplication of desired genotypes of fruit crops during *in vitro* cultures and growth regulators play a significant role in inducing axillary shoots during *in vitro* cultures. Cytokinins have been found effective in suppressing apical dominance and inducing axillary bud development. Main effect of cytokinin (BAP) upon proliferation parameters was observed significant (Table 3). Highest proliferated cultures (66.66%)

were obtained when MS medium was supplemented with 0.40 mg L^{-1} BAP. Concentrations higher or lower than this resulted in significant reduction in proliferation percentage. Lowest proliferated culture (6.66%) was observed in MS medium devoid of growth regulators (control). NAA proved inferior to BAP in inducing axillary shoot proliferation. Increasing the NAA concentration further worsened the proliferation and minimum proliferated cultures (3.33%) were recorded with NAA (0.03 mg L^{-1}). Combinations of auxins and cytokinins significantly improved explant proliferation percentage. Among nine different treatment combinations of cytokinin and auxin tried, maximum proliferation of 80% was recorded with BAP + NAA (0.40 + 0.01 mg L^{-1}) followed by 70% with BAP + NAA (0.40 + 0.02 mg L^{-1}) (Fig. 2). Similar trend was observed with other proliferation parameters. Maximum proliferation grade of 3.90 and shoot number per explant of 13.71 was observed with BAP + NAA (0.40 + 0.01 mg L^{-1}). BAP alone or in combination with NAA was most effective in improving the proliferation parameter in the present study. The observations of proliferation parameters are in close conformity to the findings many workers who used a combination of cytokinin (BAP) and auxin (NAA or IBA) for shoot proliferation and multiplication in different *Prunus* species (Gurel and Gulsen, 1998; Muna *et al.,* 1999; Chanuntapipat *et al*., 2003 and Isikalan *et al*., 2008).

Table 3: Influence of growth regulator regimes on proliferation parameters of forced explants of established cultures of almond cv. 'Shalimar'

Growth regulators (mg L^{-1})	Proliferation (%)	Proliferation grade	Number of shoots explant^{-1}
Control (0.00)	6.66 (14.76)	1.00	2.10
BAP 0.20	30.00 (33.21)	1.70	7.41
0.40	66.66 (54.75)	2.60	10.13
0.60	40.00 (39.23)	2.00	8.70
NAA 0.01	13.33 (21.34)	1.10	3.13
0.02	10.00 (18.44)	1.00	2.81
0.03	3.33 (8.61)	1.00	1.71
BAP 0.20 + NAA 0.01	36.66 (37.25)	2.53	9.81
BAP 0.40 + NAA 0.01	80.00 (63.44)	3.90	13.71
BAP 0.60 + NAA 0.01	46.66 (43.08)	3.00	10.08
BAP 0.20 + NAA 0.02	33.33 (35.25)	2.00	8.43
BAP 0.40 + NAA 0.02	70.00 (56.79)	3.36	12.41
BAP 0.60 + NAA 0.02	43.33 (41.16)	2.10	9.00
BAP 0.20 + NAA 0.03	26.66 (31.07)	1.40	6.20
BAP 0.40 + NAA 0.03	56.66 (48.83)	2.40	8.73
BAP 0.60 + NAA 0.03	36.66 (37.25)	1.90	7.76
$CD_{0.05}$	3.39	0.20	0.41

Proliferation score: 1 = poor, 2 = fair, 3 = good, 4 = excellent
Data in the parenthesis are arc sine transformed values of original percentages.

Two phase system was used for rooting of micro-shoots as this procedure had been found effective in almond by Bouza (1997), Rugini and Verma (1982) and Qadiri *et al.* (2002). The micro-shoots from proliferated cultures (10-15 mm long) were incubated in dark for 10 days on root induction media (MS medium supplemented with different concentrations of IBA) for root initiation. This was followed by the transfer of these shoots to root development medium (hormone-free MS medium) and their incubation under normal light conditions of culture room.

All the rooting parameters were significantly influenced by the supplementation of MS medium with different concentrations of IBA (Table 4). Maximum rooting of 83.33% in shoots was observed with 1.0 mg IBA L^{-1} followed by 36.66% with 0.5 mg IBA L^{-1} (Fig 3). Increasing the concentration of IBA showed negative effect on rooting percentage; and lowest rooting of 6.66% was observed with 1.5 mg IBA L^{-1}. Similar trend was observed with other rooting parameters. Maximum root number explant^{-1} (4.80) and root length (39.66 mm) was obtained with IBA @ 1.0 mg L^{-1}. Concentrations higher or lower than this had a significant reduction effect upon both these parameters. Optimum

Table 4: Influence of different concentrations of IBA on *in vitro* rooting in almond cv. 'Shalimar'

IBA concentrations (mg L^{-1})	Rooting (%)	Root number $shoot^{-1}$	Root length $shoot^{-1}$ (mm)
0.5	36.66 (37.24)	2.76	25.33
1.0	83.33 (65.96)	4.80	39.66
1.5	6.66 (14.92)	1.43	19.00
$CD_{0.05}$	4.19	0.29	3.51

Data in the parenthesis are arc sine transformed values of original percentages

concentration of auxin for the rooting of almond was found to be 1.0 mg L^{-1}, which not only yielded maximum rooting percentage and number of roots/shoot but also the maximum root length. These results are in close conformity with Bouza (1997) in *Prunus tenella*, Sharma *et al.* (1992) in colt, Qadiri *et al.* (2001, 2002) in almond and Livari and Soghadi (2005) in GF-677 almond rootstock.

Conclusions: During the present investigation a complete protocol has been standardized for the propagation of almond cv. 'Shalimar'. Forced shoot tips are the best starting explant material for plant multiplication through tissue culture. MS medium proved best for all the stages of culture. Lack of quality planting material being the main reason for the low almond production in the J&K State, therefore this technique has opened a new alternative route for *in vitro* mass multiplication of true to type and disease-free planting material of almond of an important cultivar.

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