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In vitro propagation of almond (*Prunus dulcis*) cv. Merced

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ABSTRACT

Forced and unforced shoot tips were surface sterilized with different sterilant regimes and incubated under normal culture room conditions. Surface sterilization of explants with mercuric chloride 0.1% (w/v) for 10 min. was found effective in improving culture asepsis (51.66%) and explant survival (55.00%). Higher values for both these parameters were recorded with forced explants in comparison to unforced ones. Main effect of growth regulators and media was significant on explant establishment which was maximum (66.66%) on ½ MS media containing BAP + IBA (0.50+0.01 mg/l). Callusing at the base of initiating cultures was minimum (24.58%) with BAP+IBA (0.25+0.01 mg/l). Microshoots from the established cultures were subcultured on the MS media supplemented with BAP and NAA alone or in combination for axillary shoot proliferation. Maximum proliferated cultures (86.66%) with maximum shoot number/explant (15.61) and proliferation grade (4.00) was obtained with BAP+NAA (0.40 + 0.01 mg/l). BAP was found superior to NAA during axillary shoot proliferation. Microshoots (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. Highest rooting of microshoots (93.33%) with maximum root number/shoot (5.90) and root length (43.00 mm) was obtained with IBA (1.0 mg/l).

Keywords: Almond, Growth regulators, Micro propagation, Proliferation, Rooting

Almond (*Prunus dulcis* Mill) is one of the major temperate nut crops of the world with maximum production in United States (California), Spain, Italy and Iran. Jammu and Kashmir is the main almond growing state of India with an area of 20 000 ha which comprises about 11% of total area under fruits. The temperate fruit crops are confined mostly to Western Himalayan region where lack of availability of adequate and genuine planting material has adversely affected the rapid expansion of the cultivated area under these crops. The inefficient and time consuming traditional methods of propagation are proving inadequate to cope with the increasing demand of the planting material of desired genotypes. Propagation of almond through seeds does not yield true-to-type plants and multiplication through cuttings is difficult because they contain more lignin and does not root well. The usual method of vegetative propagation of almond cultivars by nursery budding is time consuming and cumbersome.

Micropropagation is the only 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. Some work has been reported on almond in India (Qadiri *et al.* 2001) and abroad (Kester *et al.* 1986, Cidem, *et al.* 2008, Kassim *et al.* 2010), but no such studies have been carried out on the well defined almond varieties in Jammu and Kashmir state. In view of importance of almond in the state economy and need for its rapid multiplication, present studies were undertaken to standardize *in vitro* propagation protocol of almond cv. Merced a commercial cultivar well adapted to agro-climatic conditions of state.

MATERIALS AND METHODS

Present investigations were carried out in Biotechnology laboratory of Division of Pomology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir during 05-08. Forced and unforced shoot tip explants were surface sterilized with different sterilant regimes 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

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in media supplemented with different combinations of growth regulators for establishment studies. Four different types of mediam, viz Murashige and Skoog's half strength-M_0, Murashige and Skoog's full strength-M_1, Anderson's-M_2 and Tabachnik and Kester-M_3 were used in the present investigation. The *p*H of the medium was adjusted to 5.7. The cultures were incubated in the growth room. (24±1^{0}C under 16/8 photoperiod with 40±3 mol/m^2/s light intensity using 40 W fluorescent tubes). Observations with regard to established and callused cultures were made within 5 ± 1 weeks of inoculation in the establishment media.

The established explants were sub-cultured in proliferation medium within 5 ± 1 weeks of culture initiation. The MS medium supplemented with 6-Benzylaminopurine (BAP) and Naphthalene-acetic-acid (NAA) was used as basal medium during proliferation studies. Shoot production at different concentrations of BAP and NAA was recorded within 5 ± 1 weeks of culture in proliferation medium. The proliferation grade was calculated on the basis of number of shoots/explant on a four point scale, ie Grade 1 (1–4 shoots), 2(5–8 shoots), 3(9–12 shoots) and 4(13–16 shoots).

For *in vitro* rooting, well developed microshoots (10–15 mm) from the proliferating cultures were aseptically excised and cultured on MS medium supplemented with different concentrations of Indole-3-butyric-acid (IBA). Observations on number of rooted explants, number of roots/shoot and maximum root length were recorded 4 ± 1 weeks after inoculation in rooting medium. The experiments were carried out using completely randomized design with 20 explants/ treatment and replicated thrice. Data was analysed using Minitab statistical package and means were compared with LSD test. Percentage data was arc sine transformed to satisfy the assumptions of analysis of variance.

Terminal and sub-terminal dormant hardwood cuttings (15–20 cm in length and 10–15 mm in diameter) were collected from field grown 25 years old mother plants during November 2005. Cuttings were given 10 min. dip in 0.2% (w/v) captan (Captan 50 WP) solution, dried under shade for 24 hr and stored in a cold chamber (4±1^{o}C) in air tight polythene bags until use. Following one month of cold treatment, cuttings were removed from cold store. Basal ends of the cuttings were re-cut by about 1.0 cm and placed in glass jars containing distilled water and kept in incubation room maintained at 24±1^{0}C with a 16 hr photoperiod. Sprouting of buds took place after 15–20 days of transferring to incubation room (Fig 1). Shoots put forth by the sprouted buds of these almond cuttings served as forced explant source.

RESULTS AND DISCUSSION

Effect of various sterilization regimes and explant source on culture asepsis was highly significant (Table 1). Highest mean aseptic cultures to the tune of 56.66% were obtained under S_5 sterilization regime (0.1% $HgCl_2$ for 10 min. + 70% ethyl alcohol for 10 sec.) which was statically at par with 51.66% recorded under S_2 sterilization regime (0.1% $HgCl_2$ for 10 min). Lowest (18.33%) aseptic cultures were obtained with S_3 sterilization regime (70% ethyl alcohol for 10 sec.). Explant source had a significant influence upon culture asepsis. Maximum aseptic cultures (40.66%) were observed when the forced explants were utilized as against 32.66% when the unforced explants were used.

Main effect of sterilants and explant source on explant survival was highly significant (Table 1). Maximum mean survival of explants (55%) was obtained under S_2 sterilization regime where mercuric chloride was applied at 0.1% for 10 min. and minimum of 11.66% was obtained under S_5 sterilization regime in which mercuric chloride was used in combination with 70% ethyl alcohol. Using explants from different sources significantly improved explant survival. Highest mean survival of 27.33% was obtained in case of forced explants as against 22.66% when unforced explants were utilized. Though S_5 treatment resulted in maximum

Table 1 Influence of various sterilants and explant source on culture asepsis and explant survival in almond cv. Merced.

Sterilant	Culture asepsis (%)			Explant survival (%)		
	Explant source			Explant source		
	Unforced	Forced	Mean	Unforced	Forced	Mean
S_1	16.66 (23.85)	23.33 (28.77)	19.99 (26.31)	20.00 (26.56)	30.00 (33.21)	25.00 (29.88)
S_2	46.66 (43.07)	56.66 (48.84)	51.66 (45.95)	50.00 (45.00)	60.00 (50.77)	55.00 (47.88)
S_3	13.33 (21.14)	23.33 (28.77)	18.33 (24.95)	16.66 (23.85)	23.33 (28.77)	19.99 (26.31)
S_4	33.33 (35.21)	40.00 (39.23)	36.66 (37.22)	13.33 (21.14)	13.33 (21.14)	13.33 (21.14)
S_5	53.33 (46.92)	60.00 (50.77)	56.66 (48.84)	13.33 (21.14)	10.00 (18.44)	11.66 (19.79)
Mean	32.66 (34.03)	40.66 (39.27)		22.66 (27.53)	27.33 (30.46)	
CD (*P*= 0.05)	Sterilants		4.15			3.86
	Explant source		2.62			2.44
	Interaction		NS			NS

S_1 (10% sodium hypochlorite for 10 min.); S_2 (0.1% $HgCl_2$ for 10 min.); S_3 (70% Ethyl alcohol for 10 sec.); S_4 (S_1+S_3); S_5 (S_2+S_3)
Data in the parentheses are arc sine transformed values of original percentages.

aseptic cultures but the survival percentage was less because this treatment resulted in the necrosis and injury of the explants whereas S_2 treatment resulted in less percentage of aseptic cultures but the survival rate was higher. Most of the workers have found that a single sterilant is more effective than the combinations for improving explant survival. Results of the present study are in close conformity with Cidem *et al.* 2008, Kassim *et al,* 2010 and Hammerschlag 1980. Higher culture asepsis and explant survival with forced explants is attributed to the fact that forced explants were developed from the cuttings incubated in the growth chamber and hence had relatively lesser disease inoculum compared to unforced explants taken from the stock plants grown in the open field conditions. Similar results have been reported by Dalal *et al* (2000).

Explant establishment

The first step of initiating *in vitro* culture is to successfully adapt the plant tissue or explant to heterotrophic mode of nutrition. This has been termed as establishment stage . The culture establishment medium is useful for conditioning the explant and stimulating its initial growth (Fig 2). Effect of different media upon establishment of forced explants of almond was significant (Table 2). Maximum explant establishment (39.71%) was obtained when the explants were cultured in MS half strength medium, followed by 36.66% in Anderson's medium and minimum (28.32%) 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 had reported varied response of almond explants to different media during *in vitro* cultures (Bouza 1997, Ainsley *et al.* 2001, Qadiri *et al.* 2002).

Main effect of growth regulators reveals that BAP alone or in combination with IBA improved explant establishment. Optimum concentration of BAP was found to be 0.5 mg/l which yielded an explant establishment of 47.08%. Concentrations higher or lower than this significantly reduced explant establishment. Supplementation of BAP fortified media with IBA further improved explant establishment. Highest explant establishment (54.99%) was observed when the explants were cultured in medium containing BAP + IBA (0.50 + 0.01 mg/l). Interaction effects between growth regulators and media was also significant and highest explant establishment (66.66%) was recorded in ½ MS medium containing BAP + IBA (0.50 + 0.01 mg/l). Requirement of growth hormones is specific and it varies due to the endogenous levels of hormones in different tissues. Our findings are in accordance with Gurel and Gulsen (1998) who observed that shoot tips of almond established best on MS medium supplemented with BAP and IBA. Similar findings were also recorded by Ainsley *et al.* (2001) in almond.

Callusing of established cultures

Main effects of basal media on callusing at the base of initiating cultures was found significant (Table 2). Highest callusing of cultures (57.22%) was observed when the

Table 2 Influence of media and growth regulators on establishment and callusing of initiating cultures (%) in forced explants of almond cv. Merced

Growth regulator	Establishment(%)					Callusing (%)				
(mg/l)	M_0	M_1	M_2	M_3	Mean	M_0	M_1	M_2	M_3	Mean
BAP	25.00	20.00	23.33	18.33	21.66	36.66	45.00	38.33	41.66	40.41
(0.25)	(30.00)	(26.56)	(28.85)	(25.30)	(27.67)	(37.25)	(42.13)	(38.24)	(40.19)	(39.45)
BAP	53.33	45.00	51.66	38.33	47.08	55.00	63.33	58.33	60.00	59.16
(0.50)	(46.91)	(42.13)	(45.95)	(38.24)	(43.30)	(47.87)	(52.74)	(49.80)	(50.77)	(50.29)
BAP	21.66	18.33	20.00	16.66	19.16	80.00	90.00	83.33	86.66	84.99
(0.75)	(27.70)	(25.30)	(26.56)	(24.04)	(25.90)	(63.44)	(71.56)	(65.95)	(68.66)	(67.40)
BAP (0.25) +	43.33	36.66	38.33	31.66	37.49	21.66	28.33	23.33	25.00	24.58
IBA (0.01)	(41.16)	(37.25)	(38.24)	(34.23)	(37.72)	(27.70)	(32.14)	(28.85)	(30.00)	(29.67)
BAP (0.50) +	66.66	50.00	60.00	43.33	54.99	41.66	48.33	45.00	46.66	45.41
IBA (0.01)	(54.75)	(45.00)	(50.77)	(41.16)	(47.92)	(40.19)	(44.04)	(42.13)	(43.08)	(42.36)
BAP (0.75) +	28.33	25.00	26.66	21.66	25.41	63.33	68.33	65.00	66.66	65.83
IBA (0.01)	(32.14)	(30.00)	(31.07)	(27.70)	(30.22)	(52.74)	(55.77)	(53.73)	(54.75)	(54.24)
Mean	39.71	32.49	36.66	28.32		49.71	57.22	52.22	54.44	
	(38.77)	(34.37)	(36.90)	(31.77)		(44.86)	(49.73)	(46.45)	(47.90)	
CD (P = 0.05)	Growth regulators			1.28		Growth regulators			1.25	
	Media			1.05		Media			NS	
	Interaction			2.57		Interaction			2.50	

Fig 1 Forcing of dormant cuttings

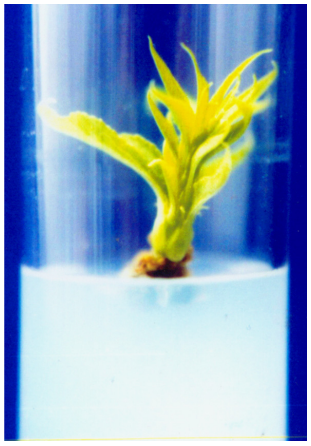

Fig 2 Explant establishment

Fig 3 Explant proliferation

Fig 4 Rooting of microshoots

explants were cultured on MS full strength medium and lowest (49.71%) when cultured on MS half strength medium.

Callusing of explants during establishment or proliferation stages is considered a negative factor and hence different growth regulator combinations/concentrations are used to minimize the phenomenon. During our study growth regulators had a significant influence on explant callusing. Minimum callusing of cultures (40.41%) was observed when the explants were cultured on medium supplemented with lowest concentration of BAP (0.25 mg/l) but increased with the increase in concentration of cytokinin and was recorded maximum (84.99%) at BAP 0.75 mg/l. Incorporation of IBA in BAP supplemented media had a beneficial influence upon reduction of callusing of cultures which was recorded minimum (24.58%) in medium supplemented with BAP+IBA (0.25+0.01 mg/l). Our findings are in close conformity with Gurel and Gulsen (1998) who observed that BA was essential for shoot development but higher levels caused callus formation which subsequently reduced the viability of the shoots. Callus formation at the base of the shoots was also observed with BA or kinetin when used at a higher concentration in Mazzard, a cherry rootstock. (Peer *et al.* 2010).

Axillary shoot proliferation

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 percentage of proliferated cultures (73.33%) was achieved when MS medium was supplemented with 0.40 mg/l BAP (Fig 3). Concentrations higher or lower than this resulted in significant reduction in proliferation percentage. Lowest percentage of proliferated cultures (10.00%) was observed in the 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 (6.66%) were recorded with NAA (0.03 mg/l). Combinations of auxins and cytokinins significantly improved explant proliferation percentage. Among nine different treatment combinations of cytokinin and auxin tried, maximum proliferation (86.66%) was recorded with BAP +NAA (0.40

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

Growth regulator	Proliferation (%)	Proliferation grade	Number of shoots/ explant
Control	10.00 (18.40)	1.00	2.75
BAP 0.20	36.66 (37.25)	2.00	8.31
BAP 0.40	73.33 (58.93)	2.93	11.45
BAP 0.60	46.66 (43.08)	2.40	9.46
NAA 0.01	16.66 (24.04)	1.26	4.16
NAA 0.02	13.33 (21.34)	1.10	3.35
NAA 0.03	6.66 (14.76)	1.00	2.23
BAP 0.20 + NAA 0.01	43.33 (41.16)	2.63	10.81
BAP 0.40 + NAA 0.01	86.66 (68.66)	4.00	15.61
BAP 0.60 + NAA 0.01	53.33 (46.91)	3.36	12.38
BAP 0.20 + NAA 0.02	40.00 (39.23)	2.20	9.15
BAP 0.40 + NAA 0.02	76.66 (61.14)	3.46	13.71
BAP 0.60 + NAA 0.02	50.00 (45.00)	2.50	10.46
BAP 0.20 + NAA 0.03	33.33 (35.25)	1.80	6.58
BAP 0.40 + NAA 0.03	66.66 (54.75)	2.53	10.20
BAP 0.60 + NAA 0.03	40.00 (39.23)	2.10	8.35
CD(P=0.05)	3.00	0.19	0.46

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

+ 0.01 mg/l), followed by 76.66% with BAP +NAA (0.40 + 0.02 mg/l). Similar trend was observed with other proliferation parameters. Maximum proliferation grade (4.00) and shoot number/explant (15.61) was observed with BAP +NAA (0.40 + 0.01 mg/l). The observations of proliferation parameters are in close conformity to the findings of a number of workers who used combinations of cytokinin (BAP) and auxin (NAA or IBA) for shoot proliferation and multiplication in different *Prunus* species. Filiz, *et al*. (2009) reported that maximum shoots/explant (11.25) were obtained from a single shoot on MS modified medium with BAP (1.0 mg/l) and IAA (0.5 mg/l).

Shoot multiplication rate of six fold was observed by Rugini and Verma (1982) on MS medium supplemented with BAP (0.7 mg/l) and NAA (0.1 mg/l) with 20 day periods of sub-culture. Highest shoot proliferation was observed in peach by Hammerschlag *et al.*(1980) in MS medium with BA + IBA (0.5 + 0.01 mg/l). Bouza (1997) reported a 3.3 fold multiplication rate in *Prunus tenella* on LP medium supplemented with BAP + IBA+ GA_3 (0.5 + 0.2 + 0.2 mg/l). Similar findings were also reported by Gurel and Gulsen (1998). In present studies a shoot multiplication rate of five fold was observed on MS medium fortified with BAP + NAA (0.40 + 0.01 mg/l). BAP alone or in combination with NAA was found most effective in improving the proliferation parameter in the present investigation. These results are in close conformity with Cidem *et al.*,2008, Kassim *et al,* 2010 who reported similar results in almond.

Rooting of microshoots

During the present study, two phase system was used for rooting of micro-shoots as this procedure had been found effective by Bouza (1997), Qadiri *et al.* (2002) and Rugini and Verma (1982) in almond. The micro-shoots (10–15 mm in length) 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 (Table 4) were significantly influenced by supplementation of MS medium with different concentrations of Indole-3-butyric acid (IBA). Maximum rooting (93.33%) of shoots was observed with 1.0 mg/l IBA (Fig 4), followed by 46.66% with 0.5 mg/l IBA. Increasing the concentration of IBA had a negative effect upon rooting percentage and lowest rooting (10.00%) was observed with 1.5 mg/l IBA. Similar trend was observed with number of roots/explant and root length. Maximum root number/explant (5.90) and root length (43.00 mm) was obtained with IBA (1.0 mg/l). Concentrations higher or lower than this had a significant reduction effect upon both these parameters. Optimum concentration of auxin for the rooting of almond was found to be 1.0 mg/l 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,* Qadiri *et al.* (2001, 2002), Ainsley *et al.* 2001 and Cidem, *et al.* 2008, in almond.

Table 4 Influence of different concentrations of Indole-3-butyric acid on *in vitro* rooting in almond cv. Merced

IBA concentration (mg/l)	Rooting (%)	Root number /shoot	Root length/ shoot (mm)
0.5	46.66 (43.07)	3.83	28.66
1.0	93.33 (77.71)	5.90	43.00
1.5	10.00 (18.43)	2.20	21.00
CD(*P*=0.05)	12.87	1.11	4.05

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

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