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RESEARCH ARTICLE

ADVENTITOUS SHOOT REGENERATION FROM IMMATURE COTYLEDONS OF PAPER SHELLED ALMOND CV. PARBAT

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ABSTRACT

The main aim of this study is to regenerate almond cultivars indirectly through callus culture that will pave way for introduction of some novel genes like spring frost resistance genes (Transgenic almond) or development of somaclonal variants that can survive in fringed areas of temperate climate for expansive cultivation. In this regard indirect regeneration potential of four thin shelled almond cultivars: Parbhat, Shalimar, Waris and Mukhdoom of J and K India was studied. The source of explants were immature almonds collected 90-120 days after pollination from orchards. The proximal ends of the immature cotyledons after chemical sterilization were cultured on MS() medium. The cultures were then maintained at 25±3°C with 16 h photoperiod(3000 lux) both in presence as well as in absence of light for first 2 weeks of culture period. After 8 weeks of culture period the explants were examined for indirect shoot production. Morphogenetic callus and the shoots regenerated from the proximal ends of these cotyledons under the influence of different phytohormones. The effects of different concentrations of 6-benzylaminopurine (BAP), Indole 3 butyric acid (IBA) and Naphthalene acetic acid(NAA) in presence or absence of dark period were determined and the best results were obtained when abaxial cotyledon surface was in contact with the culture medium.Shoot regeneration percentage was highest in both cultivars with dark treatment on Murashige and Skoogs (MS) medium at BAP(20 µM) and IBA(5 µM) concentration. BAP(5 & 10 µM) and IBA(5µM) combination significantly reduced both shoot regeneration percentage and the number of adventitious shoots produced per explant. Without dark incubation for first 2 weeks of culture period regeneration percentage was decreased in both cultivars There was also significant decrease in regeneration potential of both the cultivars at IBA(10 µM) or NAA(5 & 10 µM) in combination with BAP(5,10 &20 µM).Frequency of shoot regeneration were 80% and 60% for cultivar Waris and Mukhdoom respectively.

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INTRODUCTION

Almond is one of the tasty nuts of temperate region. Almond kernels are not only nutritious but also have medicinal properties. Each tree when grown from the seed has its unique taste and fruit characteristics with the result the uniformity of the crop is not maintained and the growers have to pack the fruits from each plant separately as single tree pack to meet the market demand. Therefore the conventional methods of almond propagation like grafting of nursery plants or seed grown plants have not met the upsurge of available market demand, as a result non conventional methods of propagation deserve their exploitation. Plant biotechnology as a tool is helpful not only in production of new genotype but have also improved the gene pool of woody fruit species. In other *Prunus* spp. including sour cherry (Hammerschlag, 1985; Cherry (Lane and Cassio, 1986; plum (Mante *et al*., 1988 &1989); Scheneider *et al*, 1992), apricot (Goffreda *et al*.1995), peach root-stock (Pooler and Scorza, 1995), ornamental cherry (Hookanson and Pooler, 2000;Yao *et al*. 2011 and Eyob K W 2017), regeneration efficiency have been improved by using the juvenile explants including cotyledons and immature embryos. Regenerations from cotyledons would not maintain clonal integrity, instead it could be used to produce somaclonal variants and provide a method for recovering transgenic almond plants. Juvenile explants being rich in natural plant hormones also respond to exogenous hormones under culture conditions as compared to adult explants which are recalcitrant to regenerate under invitro conditions. Hence the objective of this investigation was to study the morphogenetic capacity of almond immature

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cotyledon explants of two local cultivars Waris and Mukhdoom of J&K India.

MATERIALS AND METHODS

Preparation of explants (Cotyledons): Immature green almond fruits from two paper shelled cultivars viz. 'Waris' and 'Mukhdoom' were collected 90-120 days after full bloom from K D farm grown trees at Sher-i-Kashmir University of Agriculture Sciences & Technology, Kashmir (SKUAST-K) Srinagar, J&K, India. After removing hulls and shells, the kernels were subjected to chemical sterilization before culturing on growth medium under invitro conditions.Seed coats were removed and the two cotyledons separated. Embryogenic axes and the tissues immediately surrounding the embryogenic axis were excised with scalpel and discarded. These explants with abaxial side in contact with medium were then cultured on Ms medium in Borosil vials (100ml).

Preparation of Culture Medium: Ready made MS medium (Murashige & Skoog 1962) supplemented with sucrose (3.0% w/v) as carbon source and agar (0.7% w/v Agar-Agar, Sigma) as solidifying agent were used. Plant growth regulators either BAP (5, 10 and 20 µM) alone or in combination with IBA/NAA (5 and 10 µM) were added to basal medium prior to pH adjustment and autoclaving. The pH of the media was adjusted to 5.7 followed by autoclaving (120°C at 15 lb pressure for 20 minutes). Explants were then either cultured in the dark for 14 days before being exposed to light or some explants were transferred directly to light (3000 lux provided by cool white fluorescent tubes) without an initial dark period of fourteen days to asses the effect of dark period on shoot initiation. The cultures were then maintained at 25±3°C with 16 h photoperiod (3000 lux). After 8 weeks of culture period the explants were examined for shoot production.

Statistical Analysis: In a completely randomized design (CRD), the experiments were carried out three times in twentyfold replication for each studied cultivar. The frequency of regeneration was calculated as the average percentage of calli differentiating into microshoots and subjected to one way ANOVA and means were separated according to least significant difference (LSD) at $P<0.05$ level. All means are represented with mean±SE after 8 weeks of culture period.

RESULTS

Experiment No. 1: Effect of different sterilents and their duration on immature almond Kernels of cv.Waris and Mukhdoom: Kernels from immature almond (90-120 days after full bloom)were imbibed overnight in double distilled water, washed with lab detergent (cedpol) and were then surface sterilized by immersion in 0.2% (w/v) Mercuric chloride ($HgCl_2$) solution with few drops of Tween-20 (surfactant) for 10 min., followed by 3 rinses in sterile distilled water.Cotyledons were dissected transversely and only the proximal half of the cotyledons is again surface sterilized by immersion in 2.0% (v/v), sodium hypochlorite (NaOCl) solution with few drops of Tween-20 (surfactant) for 20 min., followed by 3 rinses in sterile distilled water.Various sterilizations trials performed are depicted in table 1.Sterilization frequency of 93.34 was observed when kernels were soaked in1% solution of Sodium hypochlorite(NaOCl) for 2 days followed by treatment with a solution of $HgCl_2$ (0.2 %) and NaOCl (2%) for 20 minutes.

Experiment No. 2: Effect of BAP and IBA on immature almond cotyledons cv.Waris and Mukhdoom: Completely sterilized proximal ends of cotyledons were cultured on Ms (1/2) medium supplemented with BAP(5,10 and 20 µM) and IBA (5 and 10 µM) combination, with their abaxial side facing towards medium. Regenerating cotyledons were characterized by swelling at the proximal ends after two weeks of culture under dark incubation.Callus development followed by shoot induction was greatest at the BAP (20µM) and IBA (5µM) combination. Frequency of shoot regeneration observed was 80% and 60% in cv.waris and Mukhdoom respectively (Table 2: Fig.1 & 2). Regeneration was evident after the fourth week onwards on basal medium,with multiple cluster of white or green adventitious buds on lower surface of cotyledons towards the proximal end .These buds later sprouted into long shoots without subculturing on same medium . Interspersed between these buds were found some white flat curly structures which later on metamorphosed into green leaves upon subculturing on MS basal medium. Cotyledons with little callus developed only few adventitious buds, whereas those with large callus masses formed more adventitious buds and subsequently more shoots. At lower concentration of BAP(5and10 µM) in combination with IBA(5 µM) there was significant decrease in regenration of adventitious shoot number per explants in both the cultivars. Culturing cotyledons under dark conditions for first 14 days increased the number of adventitious buds per explant. This treatment improved regeneration percentage at BAP (5,10 and 20µM) in combination with IBA (5µM) in both cultivars and simultaneously the adventitious shoot number was also increased significantly at p=0.05 level (Table 2). By increasing the IBA concentration to 10µM under these conditions both regeneration percentage and average adventitious shoot number decreased significantly(Table 3). Under these experimental conditions regeneration rates for cultivars 'Waris' and 'Mukhdoom' were 40% and 20 % respectively.

Experiment No. 3: Effect of BAP and NAA on immature almond cotyledons cv.Waris and Mukhdoom: In this experiment MS medium was supplemented with BAP(5,10 and 20 µM) and NAA (5 and 10 µM) combination and the results are presented in table 2.Cultures were also incubated in dark for first 14 days of culture period. Comparatively a low regeneration potential was observed in both the cultivars at BAP(5,10 and 20 µM) and NAA (5 and 10 µM) combination. Callus development followed by shoot induction was greatest at the BAP (20µM) and NAA (5µM) combination. Frequency of shoot regeneration observed was 40% each in cv.waris and Mukhdoom respectively (Table 2).Again regeneration was evident after the fourth week onwards on basal medium,with multiple cluster of white or green adventitious buds on lower surface of cotyledons towards the proximal end in both cultivars These buds later sprouted into long shoots without subculturing on same medium (Fig. 4 & Fig. 5).By increasing the NAA concentration to 10µM under these conditions both regeneration percentage and average adventitious shoot number decreased significantly at p=0.05 level (Table 2) in both cultivars(Fig. 6). Under such conditions regeneration rates for cultivars 'Waris' and 'Mukhdoom' were 20% and 10 % respectively.

DISCUSSION

Studies on seed sterilization have shown the maximum percentage of sterization was achieved on HgCl2 (0.1-0.2%)

Table 1. Effect of different sterilents on immature almond Kernels. Data recorded after 4 weeks of culture period

S. No.	Soaking	Sterilent "x" in 100ml of DDW + Tween-20 (0.05ml) x =	Duration in min.	**Percentage Response		
				Survival	Contamination	Sterilization
1	In water 2-d	$HgCl_2$ = 0.1 g	15	100	73.33	26.67
2	-do-	$HgCl_2$ = 0.2 g	20	100	46.66	53.34
3		*$HgCl_2$ = 0.2g followed by	10	100	60.00	40.00
	In NaOCl (0.5%) 2-d	NaOCl = 2ml	8			
4.	In NaOCl (1%) 2-d	$HgCl_2$ = 0.2 g	20	100	16.66	83.34
5	In NaOCl (1%) 2-d	$HgCl_2$ = 0.2 g	25	100	13.33	86.67
6	In NaOCl (1%) 2-d	*$HgCl_2$ = 0.2 g + NaOCl = 2ml	20	100	6.66	93.34

* *Between any two or more sterilents given separately, the explants are rinsed 3 times with autoclaved DDW.*
** *15 replicates/treatment*

Table 2.Effect of BAP, IBA and dark treatment on adventitious shoot development from immature cotyledons of four almond cultivars after 12 weeks of culture on MS (½) medium(values are mean of three replicates)

Cultivar	BAP (µM)	IBA (µM)	NAA (µM)	Dark treatment	Regeneration * %	No. of shoots ** X±S.E.	Cultivar	BAP (µM)	IBA (µM)	NAA (µM)	Dark treatment	Regeneration * %	No. of shoots ** X±S.E.
Waris	5.0	5.0		7	50.00	3±0.60	Mukhdoom	5.0	5.0		7	30.00	1±0.60
	10.0	5.0		7	60.00	5±0.10		10.0	5.0		7	30.33	2±0.10
	20.0	5.0		7	80.00	9±0.50		20.0	5.0		7	60.00	6±0.50
	5.0	5.0		—	10.00	1±0.30		5.0	5.0		—	00.00	0±0.0
	10.0	5.0		—	20.00	2±0.50		10.0	5.0		—	10.00	1±0.50
	20.0	5.0		—	30.00	3±0.30		20.0	5.0		—	10.00	1±0.30
	5.0	10.0		7	5.00	2±0.50		5.0	10.0		7	00.00	0±0.0
	10.0	10.0		7	10.00	2±0.10		10.0	10.0		7	00.00	0±0.0
	20.0	10.0		7	40.00	6±0.60		20.0	10.0		7	20.00	5±0.60
	5.0	10.0		—	00.00	0±0.0		5.0	10.0		—	00.00	0±0.0
	10.0	10.0		—	00.00	0±0.0		10.0	10.0		—	00.00	0±0.0
	20.0	10.0		—	10.00	7±0.50		20.0	10.0		—	00.00	0±0.0
	5.0		5.0	7	00.00	0±0.0		5.0		5.0	7	00.00	0±0.0
	10.0		5.0	7	10.00	2±0.10		10.0		5.0	7	10.00	2±0.10
	20.0		5.0	7	40.00	4±0.50		20.0		5.0	7	40.00	4±0.50
	5.0		5.0	—	00.00	0±0.0		5.0		5.0	—	00.00	0±0.0
	10.0		5.0	—	00.00	0±0.0		10.0		5.0	—	10.00	1±0.50
	20.0		5.0	—	10.00	1±0.30		20.0		5.0	—	10.00	1±0.30
	5.0		10.0	7	00.00	0±0.0		5.0		10.0	7	00.00	0±0.0
	10.0		10.0	7	10.00	3±0.10		10.0		10.0	7	10.00	2±0.10
	20.0		10.0	7	20.00	2±0.60		20.0		10.0	7	10.00	3±0.60
	5.0		10.0	—	10.00	1±0.80		5.0		10.0	—	00.00	0±0.0
	10.0		10.0	—	00.00	0±0.0		10.0		10.0	—	5.00	1±0.50
	20.0		10.0	—	00.00	0±0.0		20.0		10.0	—	10.00	1±0.50

* — numberof cotyledons that produced adventitious shoots. Mean values followed by different letters ar significantly different at the 0.05 probability level according to LSD. **— number of adventitious shoots per regenerating cotyledon mean value ± standard error. Treatments failingto induce adventituus shoot production (0.00) not presented.

for 10-20 min treatment (Nekrosova, 1964 in Prunus spp. and Mehra and Mehra, 1974 in almond) which is in agreement with the present studies, where 86.67% of seed sterilization was achieved on HgCl2(0.2%) for 25 min. Further increase in seed sterilization percentage, 93.34% was achieved by treating the pre-soaked seeds (in 1% NaOCl overnight) with a solution containing HgCl2 (0.2g) and NaOCl (2ml) per 100 ml, along with Tween-20 (0.05%) for 20 min. which is line with studies of Jalil *et al*., 2011 in Prunus hybrid; Manish *et al* 2016 in Clonal Cherry Rootstock and Eyobe KW 2017 in Plum, but contradict the studies of earlier workers who used ethanol (70%) for 1 min. followed by NaOCl (0.5-20%) for 10-30 min. (Hisajima, 1982 in almond; Mante *et al*., 1989 in Prunus species; Pieterse, 1989 in apricot; Pooler and Scorza, 1995 in peach and Hokanson and Pooler, 2000 in cherry; Anisley et, al 2001 in Almond; Bhagwat and David, 2004 in Sweet Cherry and Matt and Jehli 2005 in Sweet Cherry). The present study reports the indirect adventitious shoot regeneration from immature almond cotyledons. Following previously reported procedures (Mante *et al*.1989; Pooler and corza,1995 and Anisley *et al* 2001) the proximal ends of the cotyledons were selected as explants in this study. Incubation of cultures in complete darkness for a period of 7 days has been demonstrated to improve the adventitious bud formation (Predieri et, al. 1989: Korban et, al 1992 and Anisely et, al 2001; Jalil *et al* 2011 and Yao *et al* 2011). In current study the cotyledon explants were cultured under dark incubation for 14 days on MS (½) basal medium and the regeneration level upto 60-80% was achieved in both cultivars. Low regeneration percentage (10%) and subsequent morphogenesis without dark treatment from mature almond and apricot cotyledons has been reported (Mehra and Mehra 1974; Nedelcheva 1998).The highest percentage of adventitious shoot regeneration described in this study is attributed to incorporation of 14 days dark period at BAP (10 and 20µM) and IBA (5 µM) combination which is not in concurrence with the findings of other workers who also used these growth regulators, but in different concentrations in different plants (Jalil *et al* 2011 in HS314 hybrid rootstock; Manish *et al* 2016 in Colonal Cherry and Eyob KW 2017 in Plum). These observations are not in conformity with those of Lane and Cassio (1986) in apricot and cherry; Pieterse (1989) in apricot; Mante *et al*., 1989; and Schemidt and Ketzel (1993; Pooler and Scoorza,1995 and Goffreda *et al*., 1995; Anisley *et al* 2001in almond ; Bhagwat and David 2004 in Sweet Cherry; Xiaomei et, al. 2008 in black Cherry; Yao *et al* 2011 in European Plum

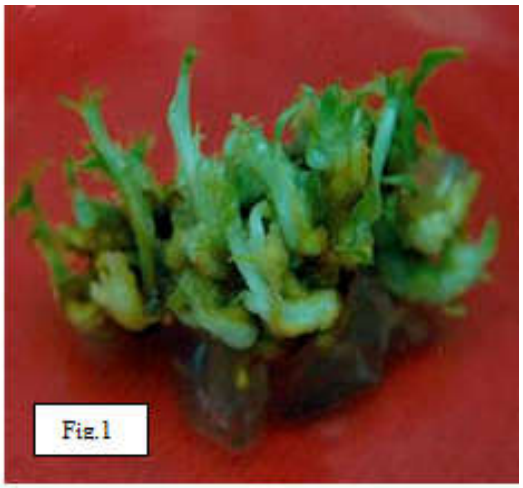

Fig 1 :- MS(½) medium containing BAP(20µM) and IBA (5µM).cv.Waris.

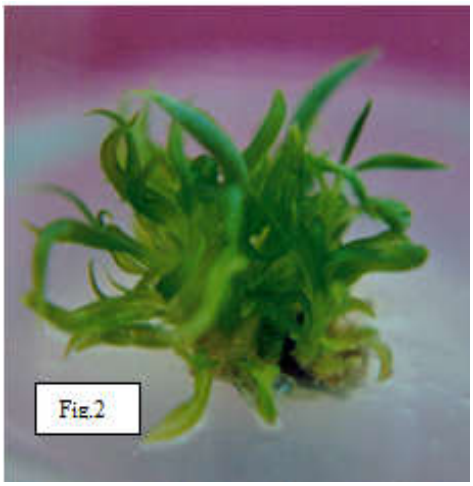

Fig 2 :- MS(½) medium containing BAP(20µM) and IBA (5µM).cv.Mukhdoom

Fig. 3. MS(½) medium containing BAP(20µM) and IBA (10µM).cv.Waris

Fig 4 :- MS(½) medium containing BAP(20µM) and NAA (5µM).cv.Waris

Fig. 5. MS(½) medium containing BAP(20µM) and NAA (5µM).cv.Waris

Fig. 6. MS(½) medium containing BAP(20µM) and NAA (10µM).cv.Mukhdoom

and Olga *et al* 2019 in apricot), who reported shoot regeneration on BAP (4.4 and 8.8 µM) in combination with either 2,4-D (1.0µM) or IAA (5.7µM).In current study increased concentration of IBA (10µM) significantly reduced the frequency of adventitious shoot regeneration in contrast with previous reports of regeneration of other *Prunus* spp. where higher levels of IBA was required for shoot regeneration from cotyledons (Mante *et al*., 1989; Pooler & Scoorza 1995 and Goffreda *et al*., 1995).There was further decrease in regeneration percentage when IBA was replaced by NAA (5 and 10) in present studies which is again in contrary to studies of Antonelli (1992) in almond; Nedelcheva (1998) in apricot; Bhagwat and David (2004) in Sweet Cherry; Matt and Jehli (2005) in Sweet Cherry; Xiaomei and Paula (2008) in black cherry and Silivia *et al* (2019) in Hansen 536 hybrid who reported higher adventitious shoot regeneration percentage under these phytohormonal combinations. Preliminary experiments with paper shelled cultivars of almond suggest that the regeneration potential is reduced with explant age (Nedelcheva 1998) and therefore the use of mature cotyledons requires further investigation before being used for

experiments with almond. However, year round experimentation has been demonstrated in ornamental cherry (Hookanson and Pooler 2000) and peach root stock (Pooler and Scoorza 1995).

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