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IN VITRO MICROROPOGATION OF SWEET CHERRY (*Prunus avium* L.) ROOTSTOCK CV. 'MAZZARD'

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ABSTRACT

The present studies on *in vitro* propagation of sweet cherry rootstock 'Mazzard' was carried out in Biotechnology laboratory, Division of Pomology, Shalimar during 2006-2009. Surface sterilization of shoot tip explants with 0.1% $HgCl_2$ for 10 minutes yielded maximum aseptic cultures and explant survival. Using explants from forced stock plant cuttings significantly improved both these parameters. Maximum explant establishment (76.0%) was observed on MS medium supplemented with BAP + kinetin (0.25 + 0.25 mg L^{-1}). The established explants were sub-cultured within 3 to 5 weeks of culture initiation for proliferation through stimulation of axillary buds. BAP + kinetin (0.25 + 0.25 mg L^{-1}) accounted for maximum proliferating cultures (99.98%) with highest multiplication efficiency in terms of proliferation grade (4.0) and shoot number explant^{-1} (16.2). Rooting experiments were carried out by incubation of micro-shoots in IBA supplemented MS media for 10 days under darkness followed by their transfer to hormone-free MS media with incubation under light conditions of culture room. Micro-shoots greater than 10 mm in length gave maximum rooting. IBA (2.5 mg L^{-1}) was found best auxin concentration which not only gave highest rooting (96.66%) but also maximized root number shoot^{-1} (6.30) and root length (48.33 mm).

Keyword: Axillary shoot proliferation, *in vitro* propagation, Mazzard, sweet cherry

INTRODUCTION

The sweet cherry is one of the important stone fruits of Jammu & Kashmir State. The increased demand of cherry fruit at national and international level has led to substantial increase in area under cherry fruit crop. This has increased the demand for quality planting material of commercial cherry cultivars. The crop yield has recently declined due to many diseases like crown and stem rot caused by different *Phytophthora* species (Mercetich *et al*., 1976). This decline is also associated with the inadaptability of current rootstocks and in particular their susceptibility to wet and heavy soil conditions. Rootstock 'Mazzard' is resistant to *Phytophthora* species and is better adapted to heavy and wet soil conditions. Conventional propagation of this rootstock through cuttings is very slow and sometimes unsuccessful because of its poor rooting capacity. Propagation through grafting or budding is a time-consuming cumbersome method. Therefore, new and rapid techniques of propagation like *in vitro* culture need to be developed for large scale multiplication of this rootstock.

Various *in vitro* studies have been conducted in Western countries for developing genotypes resistant to biotic and abiotic factors, production of haploids, germplasm exchange and preservation (Hammat and Grant, 1997; Erbenova *et al*., 2001; Dai-Hong Yan *et al.,* 2004). However, very little studies have been carried out on sweet cherry genotypes in India, particularly J&K State with respect

to *in vitro* multiplication of locally adapted genotypes. The present study was aimed at to develop a proper technique of stock plant manipulation for obtaining explants suitable for *in vitro* culture so as to standardize the micropropagation protocol of sweet cherry rootstock 'Mazzard' for commercial use.

MATERIALS AND METHODS

Studies were carried out for *in vitro* propagation of sweet cherry rootstock 'Mazzard' in Biotechnology Laboratory, Division of Pomology, SKUAST-K, Shalimar during 2006-2009. Dormant cuttings were collected from mature cherry trees, treated with Captan (@ 0.2% w/v) and stored in refrigerator in polythene bags until use. The basal ends of cuttings were re-cut by about 1 cm before use and placed in glass jars containing distilled water. The cuttings were incubated at 24±1^{o}C under normal culture room conditions for forced growth of buds. The forced explants developed from these cuttings were excised for establishment studies. The explants were cultured in four basal media supplemented with different concentrations of growth regulators viz., MS medium (Murashige and Skoog, 1962), DK medium (Driver & Kuniyuki, 1984), Knop's macro and MS organics (Snir, 1982) and woody plant medium (Lloyd and McCown, 1980). The pH of these media was adjusted to 5.7 with 0.1 N HCl/NaOH and 0.7% agar (w/v) was added as gelling agent before autoclaving at 121^{o}C for 15 minutes.

The established explants were subcultured within 3-5 weeks of culture initiation on MS medium supplemented with different concentrations of benzylaminopurine (BAP) and kinetin (0.0, 0.25, 0.5, and 1.0 mg L^{-1}) for proliferation through stimulation of axillary bud formation. Shoot production was recorded within 4 to 6 weeks of culture initiation. Microshoots from the proliferated cultures were aseptically separated and divided into four groups on the basis of shoot length. Each group was cultured on MS medium supplemented with different concentrations of indole-3-butyric acid (IBA) viz., 1.0, 2.0 and 3.0 mg L^{-1} for standardization of shoot length for rooting. Each treatment comprised of 10 explants with one explant per glass tube. Following standardization of shoot length, a wider range of IBA concentration (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}) was tried for optimizing the auxin concentration for rooting. During rooting experiments, microshoots were first cultured in root induction medium (MS supplemented with different concentrations of IBA) under dark conditions for 10 days. These shoots were then transferred to root development medium (hormone-free MS medium) and incubated under ambient conditions of culture room (16/8 hr photoperiod, 24±1^{o}C temperature, 40±3 mol m^{-2} s^{-1} light intensity using 40 W fluorescent tubes). Observations on rooting parameters were recorded 4±1 weeks after inoculation in rooting media.

Plantlets with well developed roots were removed from 6 week old cultures and washed thoroughly with double distilled sterile water to remove all traces of agar jelled media. These were planted in glass jam bottles containing a mixture of vermiculite-perlite-coco peat (1:1:1) saturated with sterile water. Lid of the bottles was closed and plants were initially kept under high humidity for 10 days under culture room conditions. Plants were fed with ¼ MS solution without organics and $CaCl_2$. The cap of the growing vessel was gradually removed to reduce the humidity. A few weeks later, when the plants began to grow, these were transferred to poly bags containing soil, vermiculite, coco peat and sand in the ratio of 8:2:2:1 for acclimatization. Data was subjected to analysis of variance using Minitab statistical package. Mean comparison was performed using least significant difference (LSD) method. Percent data were arcsine transformed before performing analysis of variance.

RESULTS AND DISCUSSION

Forced and unforced explants derived from donor stock plants were subjected to six different sterilization regimes using MS as basal medium. Effect of sterilization regimes and forcing on culture asepsis and survival was significantly high (Table 1). Maximum aseptic cultures (43.9%) were obtained under S_6 sterilization regime in which sodium hypochlorite was used in combination with fungicide and

antibiotic followed by 42.9% under S_2 (0.1% mercuric chloride) sterilization regime. Though the S_6 treatment yielded maximum aseptic cultures but the survival percentage was less (7.0%) because this treatment resulted in necrosis and injury to explants. S_2 treatment resulted in lesser percentage of aseptic cultures compared to S_6 but the culture survival was higher (51.4%). Many workers have observed a single sterilant to be more effective than the combinations for explant survival. These results are in close conformity with Dalal *et al.* (1992) in grape, Modgil *et al.* (1994) in apple, Muna *et al.* (1999) and Erbenova *et al.* (2001) in cherry. Culture asepsis and explant survival were better when forced explants were used. This may be due to relatively less inoculum load of forced explants developed from the cuttings incubated in growth chamber in comparison to unforced explants taken from field grown stock plants which are exposed to the attack of different micro-organisms.

Table 1: Influence of various sterilents and forcing upon culture asepsis (%) and culture survival (%) in sweet cherry cv. Mazzard

Sterilants	Culture asepsis (%)			Culture survival (%)		
	Forced	Unforced	Mean	Forced	Unforced	Mean
S_1	34.2 (31.6)	32.8 (29.3)	33.5 (30.5)	26.5 (31.0)	22.5 (28.3)	24.5 (30.0)
S_2	44.8 (49.6)	41.1 (43.3)	42.9 (46.5)	56.9 (49.0)	45.9 (42.6)	51.4 (45.8)
S_3	32.3 (28.6)	28.4 (22.6)	30.4 (25.6)	15.2 (23.0)	10.9 (19.3)	13.1 (21.2)
S_4	37.1 (36.3)	35.8 (34.3)	36.5 (35.3)	13.6 (21.6)	8.5 (17.0)	11.1 (19.3)
S_5	37.2 (36.6)	35.8 (34.3)	36.5 (35.3)	9.9 (18.3)	7.3 (15.6)	8.6 (17.0)
S_6	46.2 (52.3)	41.7 (44.3)	43.9 (48.3)	8.6 (17.0)	5.6 (13.6)	7.1 (15.3)
Mean	38.6 (39.2)	35.9 (34.7)		21.8 (26.6)	16.8 (22.8)	
$CD_{0.05}$						
Sterilants		0.57			1.30	
Forcing		0.33			0.75	
Sterilants x Forcing		0.81			ns	

*The values in parenthesis are transformed values ($\sin^{-1}\sqrt{p}$) of original percentage. S_1 = 10% NaOCl for 10 min.; S_2 = 0.1% $HgCl_2$ for 10 min.; S_3 =70% ethyl alcohol for 10 seconds;. $S_4 = S_1+S_3$; $S_5 = S_2+S_3$; $S_6 = S_1$+ 0.01% bavistin + 0.25% cefatoxin for 10 min

Explant establishment

The main purpose of explant establishment is to successfully adapt the plant tissue or explant to heterotrophic mode of nutrition. Culture establishment medium is useful for conditioning the explants and stimulating their initial growth. Various media viz., MS medium, DK medium, Knop's macro and M S organics medium and woody plant medium have been tried for *in vitro* studies of cherry by many workers with varying degree of success (Snir, 1982; Hammat and Grant, 1997; Erbenova *et al.*, 2001). In the present study, amongst the four media evaluated, MS medium (M_1) proved superior to others in yielding maximum explant establishment (Table 2). This was followed by DK medium (M_2) whereas low explant establishment was observed when explants were cultured in woody plant medium (M_4).

Owing to endogenous hormonal levels, the requirement of exogenous hormones varies considerably with the nature of the tissue. Exogenous application of cytokinins releases lateral buds from apical dominance (Wickson and Thimann, 1958; Sachs and Thiamann, 1964) and promotes cyto-differentiation (Fukuda and Komamine, 1985). Since BAP stimulates shoot elongation (Jones, 1967) and shoot proliferation (Pieniazek, 1968), this cytokinin has routinely been used during *in vitro* multiplication of temperate fruit and nut tree species. At times combination of cytokinins has proved beneficial (Anderson, 1983; Shen and Mullins, 1984). In present study, growth regulator combinations showed significant effect upon explant establishment with maximum explant establishment in medium supplemented with BAP + kinetin (0.25 + 0.25 mg L^{-1}) followed by medium supplemented with BAP + IBA (0.50 + 0.02 mg L^{-1}). The interaction effect of media and growth regulators on the establishment of

explants was also significant. Explants cultured in MS medium containing BAP + kinetin (0.25 + 0.25 mg L^{-1}) recorded highest explant establishment (76.0%).

Table 2: Influence of media and growth regulator regime on explant establishment (%) with forced explants of sweet cherry rootstock 'Mazzard'

Growth regulators (concentration, mg L^{-1})		Media				
		M_1	M_2	M_3	M_4	Mean
BAP	0.25	21.07 (27.33)	17.40 (24.66)	16.53 (23.99)	14.43 (22.33)	17.35 (24.58)
	0.50	41.88 (40.33)	38.46 (38.33)	36.20 (36.99)	18.75 (35.66)	33.82 (37.83)
	1.00	23.99 (29.33)	21.55 (27.66)	23.99 (27.33)	18.30 (25.33)	21.95 (27.41)
Kinetin	0.25	17.40 (24.66)	15.25 (22.99)	14.84 (22.66)	13.62 (21.66)	15.27 (22.99)
	0.50	20.13 (26.66)	16.96 (24.32)	15.68 (23.33)	11.69 (20.00)	16.11 (23.58)
	1.00	15.68 (23.33)	13.62 (21.66)	12.83 (20.99)	10.94 (19.33)	13.26 (21.33)
BAP + Kinetin	0.25+0.25	75.99 (60.66)	60.37 (50.99)	46.51 (42.99)	36.77 (37.33)	54.91 (47.99)
	0.50+0.50	30.72 (33.66)	25.16 (30.11)	24.48 (29.66)	22.50 (28.33)	25.71 (30.44)
	1.00+1.00	20.59 (26.99)	18.30 (25.33)	16.97 (24.33)	12.44 (20.66)	17.07 (24.33)
BAP+IBA	0.25+0.02	25.50 (30.33)	22.51 (28.33)	22.02 (27.99)	16.97 (24.33)	21.75 (27.74)
	0.50+0.02	43.02 (40.99)	38.44 (38.32)	36.20 (36.99)	33.80 (35.66)	37.86 (37.99)
	1.00+0.02	24.98 (29.99)	23.00 (28.66)	23.00 (28.66)	18.30 (25.33)	22.32 (28.16)
Kinetin + IBA	0.25+0.02	18.30 (25.33)	13.62 (21.66)	12.83 (20.99)	10.95 (19.33)	13.92 (21.83)
	0.50+0.02	31.25 (33.99)	27.03 (31.33)	25.50 (30.33)	21.07 (27.33)	26.21 (30.74)
	1.00+0.02	13.62 (21.66)	12.44 (20.66)	11.68 (19.99)	9.53 (17.99)	11.81 (20.08)
Mean		28.2 7 (31.68)	24.27 (29.00)	22.61 (27.81)	18.00 (25.36)	
$CD_{0.05}$	Growth regulators		1.10			
	Media		0.57			
	Growth regulator × media		2.21			

M_1 = Murashige & Skoog medium; M_2 = Driver & Kuniyuki medium; M_3 = Knops' macro- and MS micro-organics medium; M_4 = Woody plant medium; *Data in parenthesis are transformed values ($\sin^{-1}\sqrt{p}$) of original percentage.

Culture proliferation

Established cultures were transferred within 35 days of culture initiation to fresh MS medium supplemented with various concentrations of BAP for shoot proliferation. Cytokinins had a significant influence upon explant proliferation (Table 3). BAP proved superior to kinetin in improving proliferating cultures with high value in BAP (0.50 mg L^{-1}). Combined application of two cytokinins further improved proliferating cultures with maximum value in BAP + kinetin (0.25 + 0.25 mg L^{-1}). Increase in the concentration of growth regulators markedly reduced percentage of proliferating cultures and lowest values were observed in medium devoid of any growth regulator (control). Continuous presence of cytokinin in medium is of utmost importance for the formation of new shoots (Nordstrom and Eliasson, 1986). Cytokinins and their concentrations significantly influenced the shoot number explant^{-1} and proliferation grade. BAP showed superior performance than kinetin and gave maximum shoot number explant^{-1} and proliferation grade when used at a concentration of 0.25 mg L^{-1}. Combination of cytokinins proved effective and improved both these parameter. Highest number of shoots with maximum proliferation grade was obtained with treatment combination of BAP and kinetin at 0.25 mg L^{-1} each (Plate 1). Increase in cytokinin concentration exhibited a declining effect on these parameters. The results are in close conformity with Sharma *et al.* (1992) who had used combinations of cytokinins for shoot proliferation and multiplication in semi-dwarf cherry rootstock 'Colt'.

Rooting of in vitro shoots

Microcuttings taken from proliferated cultures were divided into four different groups on the basis of shoot length and transferred to the rooting media supplemented with different concentrations of Indole-3-butyric acid (IBA) to standardise the shoot length for maximising rooting of *in vitro* shoots. Initial

length of *in vitro* microcuttings had a profound effect on the rootability of shoots (Fig. 1). Shoot length of 10 mm or more resulted in maximum rooting. These results are in close conformity with Dalal *et al.* (1992), who reported 100% rooting in shoots equivalent to or exceeding 10 mm in grape vine.

After the standardization of shoot length for rooting, shoots of 10-15 mm length were cultured in IBA supplemented MS medium for root induction and kept in dark for 10 days followed by their transfer to hormone free media for root development in presence of light for 10-20 days. Two phase system for obtaining roots in micro-cuttings was followed which had been earlier used by Bouza (1997) in rooting of micro-shoots of *Prunus tenella* and Qadri *et al.* (2002) in thin shelled almond. IBA concentrations had a significant influence upon all rooting parameters (Table 4). Increase in the

Table 3: Influence of growth regulators on proliferation of forced explants of established cultures of sweet cherry rootstock 'Mazzard'

Growth regulators (concentration, mg L^{-1})		Proliferating cultures (%)	Shoot number explant^{-1}	Proliferation grade*
Control		3.85 (11.33)	3.12	1.00
BAP	0.25	86.36 (68.33)	12.32	2.96
	0.50	95.43 (77.66)	9.38	2.67
	1.00	62.65 (52.33)	6.21	1.80
Kinetin	0.25	22.51 (20.33)	5.23	2.70
	0.50	7.28 (15.66)	4.37	2.10
	1.00	4.56 (12.33)	3.80	1.10
BAP + kinetin	0.25 + 0.25	99.98 (90.66)	16.22	4.00
	0.50 + 0.25	99.91 (88.33)	13.67	3.32
	1.00 + 0.25	69.79 (56.66)	9.45	1.90
	0.25 + 0.50	97.17 (80.33)	13.52	3.72
	0.50 + 0.50	89.03 (70.66)	9.88	2.74
	1.00 + 0.50	60.37 (50.99)	7.36	2.00
	0.25 + 1.00	38.46 (38.33)	10.32	2.88
	0.50 + 1.00	67.64 (55.33)	8.92	2.62
	1.00 + 1.00	42.45 (40.66)	6.48	1.90
$CD_{0.05}$		4.67	1.98	0.36

The data in parenthesis are transformed values ($\sin^{-1}\sqrt{p}$) of original percentage;
*Proliferation grades: 1= poor, 2 = fair, 3= good, 4= excellent.

Plate 1: (Right to left) a) Forcing of dormant cuttings of mother stocks plants; b) Shoot proliferation with BAP + kinetin (0.25 + 0.25 mg L^{-1}); c) Rooting of plantlets in MS medium supplemented with IBA (2.5 mg L^{-1}); d) Hardening of plantlets in glass containing vermiculite, perlite & coco-peat (1:1:1)

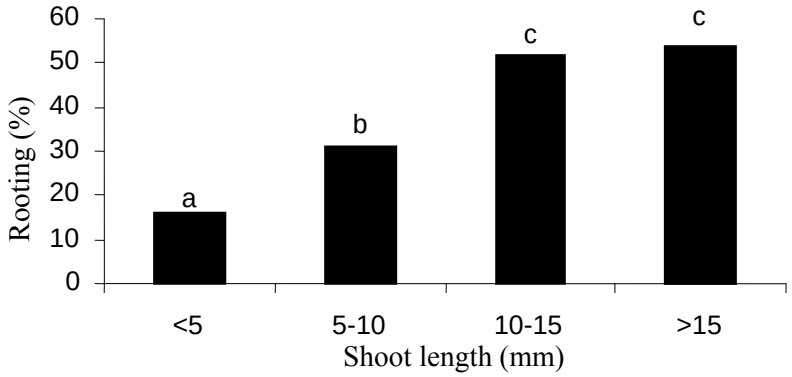

Fig. 1: Influence of shoot length on rooting of micro-shoots of sweet cherry cv Mazzard. Columns with different letters are significantly different at $P_{0.01}$ with LSD test. Data in the columns is averaged over IBA concentrations.

Table 4: Influence of IBA concentrations on *in vitro* rooting of micro-shoots of sweet cherry rootstock 'Mazzard'

IBA (mg L^{-1})	Rooting (%)	Root number/shoot	Root length (mm)
0.50	08.33 (16.70)	3.30	28.33
1.00	46.66 (43.08)	3.56	30.00
1.5	60.66 (51.27)	3.60	38.33
2.00	70.33 (57.19)	4.20	40.66
2.50	96.66 (83.85)	6.30	48.33
3.00	50.33 (45.19)	3.10	21.66
$CD_{0.05}$	11.21	0.32	3.13

*Data in parenthesis are transformed values ($\sin^{-1}\sqrt{p}$) of original percentage.

concentration of IBA improved rooting. Maximum rooting (96.66%), root number $shoot^{-1}$ (6.30) and root length (48.33 mm) was recorded with 2.5 mg L^{-1} IBA. Concentration higher than this had a negative effect upon rooting parameters. Rootstock 'Mazzard' has a low inherent rooting capacity and its propagation through cuttings have been found unsuccessful (Feucht and Dausend, 1976: Hartmann *et al*. 1997). Makunda *et al*. (1988) failed to induce rooting in microshoot during *in vitro* culture of cherry root stock 'Mazzard' through the use of 1-2 mg L^{-1} IBA. However, we were successful in obtaining high percentage of rooting in Mazzard. This may be effect of explant source as we used forced explants in the present study. Rooted plantlets were hardened in glass jam bottles containing vermiculite, perlite and coco-peat (1:1:1) with 92% success.

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