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Callus induction and adventitious shoot regeneration in two genotypes of sweet cherry (*Prunus avium*)

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ABSTRACT

Studies were carried out during 2006–09 with two genotypes of sweet cherry–Mazzard (rootstock) and Bigarreau Noir Grossa (commercial cultivar) for developing adventitious shoot propagation protocol using *in vitro* leaf segments as explants. MS medium supplemented with BAP + 2,4-D (0.50 + 2.0 mg/l) resulted in maximum callus initiating cultures (66.19%) with maximum callus fresh weight (2.85 g). Highest adventitious shoot initiating cultures (32.74%) with maximum shoot number/culture (9.71) was recorded with BAP + NAA (2.0 + 1.0 mg/l). Adventitious shoots (10 mm or more) cultured in root induction medium (MS medium supplemented with IBA) were incubated under darkness for 10 days and then transferred to root development medium (hormone-free MS medium) under normal incubation conditions of culture room (16/8 hr photoperiod, 24±1 °C temperature, 40±3 mol/m²/s light intensity using 40 W fluorescent tubes). Maximum rooting in microshoots (36.15%) with maximum number of roots/shoot (9.58) were recorded with IBA (2.5 mg/l). Among the two genotypes, Mazzard proved superior to Bigarreau Noir Grossa with respect to all shoot and root regeneration parameters.

Key words: Bigarreau Noir Grossa, Callus organogenesis, Mazzard, Micropropagation, Sweet cherry

Plant regeneration from *in vitro* callus cultures can play an important part in the propagation and improvement of crop plants. Cell and callus cultures are prerequisites for improvement of crops through genetic engineering and gene transfer methods represent a powerful tool for the production of genetically improved plants. Genetic improvement of cherry through conventional breeding methods is limited by high heterozygosity, polyploidy and long juvenile period. Genetic engineering through tissue culture technology provides an opportunity for overcoming these restrictions.

Most of the *Prunus* species are recalcitrant to *in vitro* adventitious shoot regeneration and only few reports are available on organogenesis from mature explants (Matt and Jehle 2005). According to Jones *et al.* (1984) there is greater possibility of promotion of adventitious shoot induction in callus derived from *in vitro* propagated plants. Shoot regeneration was obtained in callus from stem sections and shoot tips of micropropagated plants of three commercial cherry cultivars (Feeney *et al*. 2007).

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Callus cultures have been found useful for non-conventional techniques of crop improvement like protoplast fusion and genetic transformation. Induction of somoclonal variation through callus organogenesis has also been used for the improvement of different horticultural crops. Development of new variants following irradiation of callus and its subsequent organogenesis has been found very effective technique in crop improvement. Keeping in view the importance of callus organogenesis, present studies were conducted for development of callus derived adventitious shoot proliferation protocol in cherry using *in vitro* leaf segments of two genotypes of sweet cherry (*Prunus avium* L.) – Mazzard and Bigarreau Noir Grossa.

MATERIALS AND METHODS

Studies were carried out in Biotechnology Laboratory, Division of Pomology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar during 2006–09 with two genotypes of sweet cherry Mazzard (rootstock) and cv. Bigarreau Noir Grossa. MS medium was supplemented with growth regulators for callus induction and shoot regeneration. For callus induction, leaf segments (1.0 cm²) from *in vitro* shoots were inoculated on MS medium containing 20.0 g/l sucrose and 7.0 g/l agar. Medium was

supplemented with different combinations of BAP (0.5 mg/l), NAA (0.5, 1.0, 2.0 mg/l) and 2,4-D (0.5, 1.0, 2.0 mg/l). The *p*H of the medium was adjusted to 5.7 with the addition of 1N HCl/NaOH and 10 ml quantity of medium was poured into 15 mm × 150 mm test tubes closed with non-absorbent cotton plugs. All medium constituents were sterilised by autoclaving at 1.06 kg/cm^2 pressure and 121 ^{o}C temperature for 20 min.

Callus induction, fresh weight and growth were recorded after 35 days of culture under normal culture room conditions (16/8 hr photoperiod, 24±1 °C temperature, 40±3 mol/m^2/s light intensity from 40 W fluorescent tubes).

Five week old nodular compact callus from each genotype was divided into small pieces of about 3.0 cm diameter and cultured on shoot induction MS medium supplemented with the following combinations of BAP (0.5, 1.0, 2.0 mg/l) and NAA (0.10, 0.25, 1.0 mg/l) for organogenesis. Number of adventitious shoots/explant was recorded after two months of culture on MS medium. Adventitious shoots were maintained on the same medium and transferred to fresh medium after every three to four weeks.

To study the effect of auxin on root initiation from regenerated shoots, elongated adventitious shoots (10 mm) from the proliferated cultures were aseptically separated and inoculated on MS medium supplemented with different concentrations of IBA (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l). Each treatment comprised 10 explants with one explant per test tube. 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 normal conditions of culture room. Observations on rooting parameters were recorded 5±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. Three 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 1:1:1:1 for acclimatization.

Data was subjected to analysis of variance using Minitab statistical package. Mean comparison was performed using Least Significant Difference (LSD) method. Per cent data were arcsine transformed before performing analysis of variance.

RESULTS AND DISCUSSION

Leaf segments from *in vitro* grown shoots of two genotypes of sweet cherry-Mazzard (rootstock) and Bigarreau Noir Grossa (commercial cultivar) were used for initiation of callus on MS medium supplemented with growth regulator combinations. Initiation and growth habit of callus was recorded after 35 days of incubation under standard culture room conditions.

Callus initiation and fresh weight

Main effect of growth regulators and genotype on callus initiation was significant (Table 1). 2,4-D was found more effective than NAA for callus induction and growth. Maximum callus initiating cultures (66.19%) were recorded with BAP+2,4-D (0.50 + 2.0 mg/l), followed by 63.11% with BAP+2,4-D (0.50 + 1.0 mg/l) and minimum of 41.07% with BAP+NAA (0.50 + 0.5 mg/l). Amongst genotypes, Mazzard rootstock proved far superior recording 59.25% callus initiating cultures compared to 54.38% in Bigarreau Noir Grossa. Callus initiation started within one week from the injured portion of explants. The growth of callus was slow in initial stages but after a fortnight it proliferated very fast. Sub culturing of callus was done on MS medium supplemented with BAP+2,4-D (0.50 + 2.0 mg/l) after 35 days. Since the growth of callus was normal on MS medium, therefore, no other medium was tried for maintenance of callus. Compact callus was produced in the callus initiation phase and it turned into nodular callus after second or third subculture. The colour of callus changed to dark brown if not subcultured at the end of 6 weeks.

Main effect of growth regulators on callus fresh weight was significant. Increasing the concentration of auxins had a positive influence upon this parameter (Table 2). Maximum

Table 1 Effect of genotype and growth regulator on callus induction (%) from *in vitro* leaf explants of sweet cherry

Growth regulator	Concentration (mg/l)	Genotype		Mean
		Mazzard	Bigarreau Noir Grossa	
BAP+NAA	0.5+0.5	42.54	39.59	41.07
BAP+NAA	0.5+1.0	57.29	50.94	54.11
BAP+NAA	0.5+2.0	61.67	56.36	59.02
BAP+2,4-D	0.5+0.5	59.92	54.84	57.38
BAP+2,4-D	0.5+1.0	65.60	60.63	63.11
BAP+2,4-D	0.5+2.0	68.49	63.89	66.19
Mean		59.25	54.38	
CD (P =0.05)				
Growth regulator	1.87			
Genotype	1.08			
Interaction	NS			

Table 2 Effect of genotype and growth regulator on callus fresh weight (g) from *in vitro* leaf explants of sweet cherry

Growth regulator	Concentration (mg/l)	Genotype		Mean
		Mazzard	Bigarreau Noir Grossa	
BAP+NAA	0.5+0.5	2.43	2.46	2.44
BAP+NAA	0.5+1.0	2.61	2.66	2.64
BAP+NAA	0.5+2.0	2.68	2.73	2.71
BAP+2,4-D	0.5+0.5	2.02	2.07	2.04
BAP+2,4-D	0.5+1.0	2.78	2.82	2.80
BAP+2,4-D	0.5+2.0	2.83	2.88	2.85
Mean	2.56	2.61		
CD (P =0.05)				
Growth regulator	0.18			
Genotype	NS			
Interaction	NS			

fresh weight of callus (2.85 g) was observed when the explants were cultured on MS medium supplemented with BAP+2,4-D (0.50 + 2.0 mg/l) and minimum (2.04 g) with BAP+ 2,4-D (0.5+0.5 mg/l). Influence of genotypes upon callus fresh weight was non-significant. The results are in conformity with those of Shi *et al.* (2006).

Adventitious shoot regeneration

Selection of right type of explant is very critical for establishing embryogenic callus cultures. Immature young parts of plants like immature embryos, young leaves/petioles, cotyledons and hypocotyls from young seedling have been successfully used as explants for producing embryogenic callus (Canli and Tian 2008, Bhagwat and David 2004, Meneghelli 2002). Organogenesis in callus cultures for shoot and root initiation can be regulated by a subtle ratio between auxin and cytokinin in the medium. Higher ratio of cytokinin to auxin has been generally found effective for shoot induction and vice versa. In the present study, young leaf segments from *in vitro* leaves were used for callus induction and the callus mass derived was cut into uniform pieces and subcultured on callus regeneration media consisting of MS medium fortified with cytokinin and auxin in different ratio. First callus increased in volume with subsequent regeneration of clusters of shoot bud primordia after 21 days. Combinations of BAP and NAA had a significant influence on adventitious shoot regeneration (Table 3). Maximum adventitious shoot regeneration (32.74%) was obtained with BAP + NAA (2.0 + 1.0 mg/l) and minimum (23.51%) with BAP+NAA (1.0 + 0.25 mg/l). Mazzard recorded higher (29.17%) adventitious shoot regeneration compared to 25.12% observed in Bigarreau Noir Grossa. Interaction between growth regulators and genotype was significant recording maximum shoot regeneration (35.22%) in Mazzard with BAP + NAA (2.0 + 1.0 mg/l). Similar trend was observed with shoot number/

Table 3 Effect of genotype and growth regulator on adventitious shoot regeneration (%) from *in vitro* leaf explants of sweet cherry

Growth regulator	Concentration (mg/l)	Genotype		Mean
		Mazzard	Bigarreau Noir Grossa	
BAP+NAA	0.50+0.10	26.98 (20.60)	23.41 (15.80)	25.19 (18.20)
BAP+NAA	1.00+0.25	25.31 (18.30)	21.71 (13.70)	23.51 (16.00)
BAP+NAA	2.00+1.00	35.22 (33.30)	30.25 (25.40)	32.74 (29.35)
Mean		29.17 (24.06)	25.12 (18.30)	
CD (P =0.05)				
Growth regulator	0.18			
Genotype	0.14			
Interaction	0.25			

Data within parenthesis are arc sine transformed values of original percentages.

Table 4 Effect of genotype and growth regulator on number of shoots/culture from *in vitro* leaf explants of sweet cherry

Growth regulator	Concentration (mg/l)	Genotype		Mean
		Mazzard	Bigarreau Noir Grossa	
BAP+NAA	0.50+0.10	6.74	6.49	6.62
BAP+NAA	1.00+0.25	5.67	4.83	5.25
BAP+NAA	2.00+1.00	10.33	9.08	9.71
Mean		7.58	6.80	
CD (P =0.05)				
Growth regulator	0.713			
Genotype	0.582			
Interaction	NS			

culture (Table 4). The maximum number of adventitious shoots (9.71) were obtained with BAP + NAA (2.0 + 1.0 mg/l), followed by 6.62 with BAP + NAA (0.50 + 0.10 mg/l) and minimum (5.25) with BAP + NAA (1.0 + 0.25 mg/l). Mazzard was found superior in terms of shoot regeneration, giving 7.58 shoots as compared to 6.80 shoots regenerated in Bigarreau Noir Grossa.

Results of present investigation revealed that BAP + NAA (2.0 + 1.0 mg/l) was the optimum combination of plant growth regulators for regeneration of shoots from the *in vitro* leaf derived callus of sweet cherry. The present results are in close conformity with the findings of Tang *et al.* (2002) who obtained adventitious shoots from sweet cherry leaf explants using BAP at 2.0 mg/l plus NAA at 0.5–1.0 mg/l. Matt and Jehle (2005) and Bhagwat and David (2004) also regenerated plants from leaf segments of sweet cherry cultivars.

Rooting of in vitro *adventitious shoots*

The formation of adventitious roots on a microcutting is a crucial step in commercial micropropagation. The type of root system formed *in vitro* depends on various factors including physical characteristics of the rooting environment, genotype quality of the microcutting etc. Two phase system for obtaining roots in microshoots was followed which had been earlier used by Bouza (1997) in rooting of microshoots of *Prunus tenella* and Qadri *et al.* (2002) in thin shelled almond. Microshoots of uniform length were cultured in root initiation medium (MS medium supplemented with different concentrations of IBA) and incubated under darkness under culture room conditions for first 10 days and then transferred to root development medium (hormone-free MS medium) under normal incubation conditions of culture room. Root induction was observed within two weeks and most of the roots had regenerated after 4 weeks. Concentration of IBA had a significant effect on rootability of microshoots of both the genotypes of sweet cherry (Table 5). The highest root regeneration (36.15%) was obtained in microshoots cultured in root induction MS medium supplemented with IBA (2.5 mg/l). The minimum root induction of 15.18% was recorded with IBA (0.50 mg/l). Effect of genotype on root induction was significant. Root induction was more with microshoots of Mazzard (28.96%) compared to 26.86% recorded with Bigarreau Noir Grossa. Root number/shoot showed a similar trend (Table 6). It also increased with increasing the auxin concentration and reached maximum (9.58) with IBA (2.50 mg/l) and then declined. Minimum root number/shoot (5.73) was recorded with IBA (0.50 mg/l). These results are in close conformity with Bouza (1997) in *Prunus tenella*, and Qadri *et al*. (2004) in almond. Rooted plantlets were first hardened in glass jam bottles containing vermiculite, perlite and cocopeat (1:1:1) under normal incubation conditions and then transferred to polybags containing soil, sand, compost under green house conditions with 60% success.

Table 5 Effect of genotype and indole-3-butyric acid on root regeneration (%) in adventitious shoots of sweet cherry

IBA concentration (mg/l)	Genotype		Mean
	Mazzard	Bigarreau Noir Grossa	
0.50	16.33 (7.94)	14.02 (5.92)	15.18 (6.93)
1.00	23.04 (15.35)	21.49 (13.45)	22.27 (14.40)
1.50	30.24 (25.40)	26.97 (20.60)	28.61 (23.00)
2.00	33.98 (31.28)	33.57 (30.61)	33.78 (30.94)
2.50	37.60 (37.26)	34.70 (32.44)	36.15 (34.85)
3.00	32.55 (28.97)	30.39 (25.63)	31.47 (27.30)
Mean	28.96 (24.36)	26.86 (21.44)	
CD (P =0.05)			
IBA	1.03		
Genotype	0.59		
Interaction	NS		

Table 6 Effect of genotype and Indole-3-butyric acid on root number/shoot in adventitious shoots of sweet cherry.

IBA concentration (mg/l)	Genotype		Mean
	Mazzard	Bigarreau Noir Grossa	
0.50	5.73 (1.00)	5.73 (1.00)	5.73 (1.00)
1.00	7.48 (1.70)	6.54 (1.30)	7.01 (1.50)
1.50	8.32 (2.10)	8.12 (2.00)	8.22 (2.05)
2.00	9.45 (2.70)	8.52 (2.20)	8.98 (2.45)
2.50	9.90 (2.96)	9.27 (2.60)	9.58 (2.78)
3.00	8.32 (2.10)	7.91 (1.90)	8.12 (2.00)
Mean	8.20 (2.09)	7.68 (1.83)	
CD (P =0.05)			
IBA	0.24		
Genotype	0.14		
Interaction	0.34		

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