IN VITRO PROPAGATION OF AMBRI APPLE, A HIGHLY PRIZED CULTIVAR OF KASHMIR HIMALAYA

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ABSTRACT

Shoot tips of Ambri cultivar of apple (*Malus pumila),* obtained from young twigs of mature trees, were used as explants to evaluate the effect of various growth regulators either separately or along with phloroglucinol supplemented in MS (1/2 strength) (1962) nutrient medium. Of different growth adjuvants used, the explants responded best to BAP (5µM) + PG (10µM) which was evinced by direct multiple adventitious shoot regeneration at the base of the explant. The shoot lumps were subcultured 3-4 times, at an interval of 4 – 6 weeks, to increase the number of regenerated shoots. Each isolated shoot was subcultured under the influence of a number of root inducing hormones. The best rooting response was scored on MS (½) +IBA (2.5µM). The plantlets thus obtained were transferred to pots for hardening.

Key words: *In vitro*, Ambri apple, mature trees, shoot tips, multiple adventitious shoots, plantlets.

Abbreviations: MS – Murashige and Skoog; BA – Benzyl adenine, PG – Phloroglucinol, IBA- Indole butyric acid, IAA – Indole -3 acetic acid, NAA- Nephthalene acetic acid, 2,4-D – 2,4-dichlorophenoxy acetic acid, Kn – kinetin, PVP-polyvinyl pyrollidine TDZ- Thidiazuron.

INTRODUCTION

Apple is the most important fruit of the temperate regions grown all over the world for its high economic value. The old saying 'an apple a day keeps the doctor away' has been reconfirmed as skin of apple fruit has been found to contain chemicals which reduce the risk of hypertension by 32% and that of cancer by 20% (Boyer and Liu, 2004; Zhul *et al*., 1999).

Apple is a dicotyledonous, low spreading woody plant belonging to family Rosaceae with subfamily Pomideae having basic chromosome number 17. The genus includes about 35 species and the cultivated apple is the result of intercrossing among various species of *Malus* (Zimmerman, 1984). Cultivated apple varieties are generally diploid but many varieties are triploid and few are tetraploid (Kochhar, 1981).

Apple is believed to have originated in Caucasus Mountains of Western Asia where vast forests of wild apple trees exist even today (Kochhar, 1981). It is the premier table fruit of the world and has been under cultivation since earliest times. Apple growing regions occur throughout the temperate zones of the world. In India the major apple producing regions include Kashmir, Himachal Predesh, Utter Predesh, Kumaon, Assam and Nilgiri Hills. Kashmir is the leading apple producing state in India with annual production of 60% of the total production in India (Anonymous, 1996). The state has remained popular for its indigenous Ambri cultivar of apple from remote past.

Ambri apple is a highly prized cultivar of Kashmir because of its longer shelf life due to thick skin (high water retention capacity), pleasant taste and good flavour. However, it has low yield and growers being money minded, have stopped its cultivation which has led to drastic decline in its production and as such its existence is under threat.

Traditional method of propagation for apple is time consuming, laborious and

involves a lot of cost besides being skilful. In view of these problems, use of newer techniques for producing large number of plants in lesser time, are preferred. Tissue culture technique seems to be the logical method for the production of self rooted clonal trees as it has the potential to provide large number of plants in less time (Kumar and Kumar, 1998). Till date there is only a recent report by Sharma *et al* on micropropagation of Ambri cv of apple from J & K state. Present work thus represents an attempt on *in vitro* propagation of Ambri cultivar of apple in the valley in its native place.

MATERIAL AND METHODS

Shoot apices obtained from young twigs of mature fruit bearing trees (50-60 year old), growing in Dhar farm at Zakura, were thoroughly washed under tap water using labolin (5%) and a wetting agent Tween-20. This was followed by their sterilization with sodium hypochlorite (10%) for 20 minutes and three times rinsing by double distilled water. After initial sterilization, these were placed in 10µM Kn solution in flasks for 24 hours at 4^{o}C (in refrigerator) with their mouths sealed, after which these were re-sterilized using $HgCl_2$ (0.1%) for 90 seconds on the hood of Laminer air flow cabinet. The traces of $HgCl_2$ were removed from the explants by rinsing with autoclaved water several times, after which they were cut to excise shoot apices of 0.5-1.5cm size for inoculation on MS nutrient medium supplemented with different phytohormones and PG in different combinations. The cultures were placed in incubation room where temperature was maintained between 22-28^{o}C with light intensity of 3000 lux maintained for 18 hours daily.

Regular transfer of explants onto fresh nutrient media was followed at least 3-5 times for controlling browning of the medium, while the culture products obtained were subcultured after every 4- 6 weeks. The complete plantlets obtained were finally transferred from culture vials to small pots for hardening.

OBSERVATIONS

Shoot apices (0.5-1.5 cm long) were cultured under the influence of various auxins, cytokinins either separately or in combinations with PG showed various degrees of response (Table-1). Best response in terms of shoot multiplication and proliferation was scored when medium was supplemented with BA (5µM) + PG (10µM). Initially the response was poor due to phenolic exudations, which was overcome by 3-5 times regular transfer of explants on fresh medium of same composition for 7-10 days. Culture establishment and shoot induction started after second week and fifth week of culture period respectively (Fig.1). This was followed by direct multiple adventitious shoot regeneration at the base of the explant (Fig-2). The adventitious shoots thus produced were subcultured in lumps after regular intervals on same medium which continued to prolifer and thus increased their number to about 1400 in six months with an average shoot number of 350 per subculture (Fig-3). During this period microshoots showed elongation as well. Elongated micro-shoots were then separated carefully and subcultured on different rooting media (Table-2). Best response was observed on MS (1/2) fortified with IBA (2.5µM) and PG (10µM) where percentage of rhizogenesis was recorded to be100. When the concentration of IBA was increased to 5µM, percentage of response got reduced drastically to 30. With IBA (2.5µM) root initials were seen in 2nd week of subculture and profuse rooting was observed after six weeks of culture period. Complete plantlets worth transplantation were recovered in 8-10 weeks (Fig-4). When the shoots were subcultured under the influence of triple auxin combination (IAA + IBA + NAA – 1 or 5µM each) either with PG (10µM) or without PG, roots started callusing immediately after initiation. Rooted shoots (5-

8cm long) were later on properly deflasked and transferred to pots containing sand- soil mixture of 1:1 for acclimatization under high humidity (Fig-5).

Table 1: *In vitro* response of the shoot apices from mature trees of Ambri cv. of apple to phytohormones.

Culture medium MS (1/2) (µM)	Response [a]	Degree of callus formation	Percentage response		Ave no of shoots / explant[b]
			Callogenesis	Caulogenesis	
BA (5)	Callus at cut end	++	10	--	--
BA (5) + PG (10)	Adventitious shoot proliferation	--	--	40	350
NAA (5)	Callus at cut end	+	05	--	--
NAA (5) + PG (10)	Callus at cut end	+	05	--	--
IAA (5)	No response	--		--	--
IAA (5) + PG (10)	No response	--		--	--
IBA (5)	No response	--		--	--
IBA (5) + PG (10)	No response	--		--	--
NAA (5)	Callus at cut end	++	10	--	--
NAA (5) + PG (10)	Callus at cut end	++	10	--	--
2,4-D(1)	No response	--	--	--	--
2,4-D(5)	No response	--	--	--	--
2,4-D(5) + PG (10)	Callus at cut end	++	--	--	--
MS basal+ PG (10)	No response	--	--	--	--

[a] 10 replicates per treatment
[b] data scored after six weeks
+ low callus, ++ moderate callus

Table 02: *In vitro* response of sub-cultured shoots to auxins for rooting.

Culture medium MS (1/2) µM	Response [a]	Degree of callus formation	Percentage response [b]	
			Callogensis	Rhizogenesis
IAA (1)	No response	--		--
IAA (5)	Callus formation	+	20	--
IAA (5) + PG(10)	Callus formation	+	20	--
NAA (1)	No response	--		--
NAA (5)	Callus formation	++	30	--
NAA (5) + PG(10)	Callus formation	++	30	--
IBA (1)	No response	--	--	--
IBA (2.5) +PG(10)	Normal direct rooting {after six weeks}	--	--	100
IBA (5) + PG(10)	Normal direct rooting (after six weeks}	--	--	30
BA (5)+IBA (1) + PG(10)	Rooting followed by callusing	++	20	40
2,4-D (5)	No response	--	--	--
2,4-D (1)	No response	--	--	--
2,4-D (5)+ PG(10)	No response	--	--	--
IAA + IBA + NAA (1µM each)	Root initiation immediately followed by callusing	+	20	20
IAA + IBA + NAA (5µM each)	Root initiation immediately followed by callusing	+++	40	40
IAA + IBA + NAA (5µM each) + PG(10)	Root initiation immediately followed by callusing	++	50	50
MS Basal	No response	--	--	--

[a] 10 replicates per treatment
[b] data scored after eight weeks unless otherwise mentioned
+ low callus, ++ moderate callus, +++ High callus

DISCUSSION

The principal objective of present investigation was to stimulate multiple shoot formation from shoot apices of mature trees of Ambri apple to obtain clonal plantlets. To initiate the work on apple culture, Zimmerman (1984) suggested use of calcium hypochlorite (20%) for controlling microbial contamination in apple cultivars but in present work, initial treatment with sodium hypochlorite (10%) for 20 minutes followed by treatment with $HgCl_2$ (0.1%) for 90 seconds (after 24 hour chilling) was found to be highly effective.

In apple, browning of explants due to phenolic exudations has been found to be a main problem during culture establishment (Jones, 1967). This problem has been overcome by the use of PVP (Walkey, 1972) or PG (Jones and Hatfield, 1976) in the medium. In present investigation, overnight chilling of explants at 4^oC in Kn (10µM) after initial sterilization, regular transfer (3-5 times) onto fresh nutrient medium of same composition and use of PG (10µM) in culture medium have been found to be effective steps for controlling browning.

Establishment and proliferation of adventitious multiple shoots from shoot apices of M.7 and M.26 apple on Quoirin's medium (1974) using vitamins of Wetmore and Sorokin (1955) enriched with floridzin and PG (10^{-3}M), BA (0.5 mg/l), IBA (1mg/l) and IAA (1mg/l) was recorded by Jones (1976). In present work, PG (10µM) and BA (5µM) in MS (1/2) medium were found to be very effective for multiple shoot initiation. In contrast, Sharma *et al* (2004) have found TDZ more effective than BA on ambri cv. Auxins like IAA, 2,4-D and IBA were found to have inhibitory effect on caulogenesis and same observations have already been reported by Snir and Erez (1980) on three apple cultivars (MM.104, MM.106 and M.109) which confirm the present studies.

Strength of medium salts has been found to play vital role in culture establishment and shoot proliferation in M.7 apple root stock and half strength MS medium seems to be most effective (Werner and Roe, 1980; Bartish and Korkhovoi, 1997). Cheema and Sharma (1983) have, however, observed that half strength MS medium favoured the development of highly hydrated shoots, which were sensitive to injury and initiated much basal callusing. Such an observation has not been recorded in present studies using MS medium with ½ salt strength but instead our observations are in line with Werner and Roe (1980) and Bartiah and Korkhovoi (1997) in finding such a medium effective for culture establishment and shoot proliferation of apple. In contrast to this Cheema and Sharma (1983) have shown that MS medium with full strength of salts fortified with BA (1.0mg/l) + IBA (0.2 mg/l) supported growth of shoot apices in apple which is supported by Sharma et al (2004) who have used TDZ in addition to BA.

In present investigation, best root initiation and elongation to obtain complete plantlets, was observed on MS (1/2) supplemented with IBA (2.5µM) + PG (10µM) which corroborates with the results of Zimmerman (1984); James and Thurbon (1979, 1981); Hicks, (1987);


Fig-1


Fig-2


Fig-3 (a)

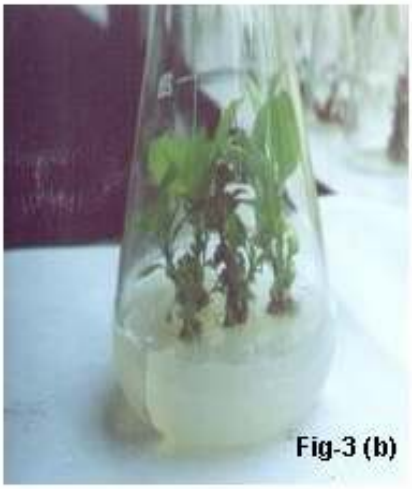
Fig-3 (b)


Fig-4


Fig-5

Fig. 1-5: Morphogenetic response of shoot apices from mature trees of Ambri cv. of apple (*Malus pumila*) to phytohormones

Fig-1 Shoot induction from mature shoot apex on MS (1/2) + BA (5µM) + PG (10µM) (after 2th week)
Fig-2 Direct multiple adventitious shoot formation on MS (1/2) + BA (5µM) + PG (10µM) (after 5th week)
Fig-3 Subcultured multiple shoots on MS (1/2) + BA (5µM) + PG (10µM) (after six months)
Fig-4 Rooting of subcultured shoots on MS (1/2) + IBA (2.5µM) + PG (10µM) (after six weeks)
Fig-5 Deflasked plantlet in Sand-soil mixture 1:1 for acclimatization.

Correa *et al.* (1990); Nui *et al.* (1995) and Puntae and Martin (1997).

There has been a long controversy over the impact of PG on rooting. Zimmerman and Broome (1981) observed that phloroglucinol (a phenolic compound) favours rooting and reduces callus formation in apple cultivar Spartin, with different concentrations of IBA (0-4.9 µM). A number of workers have reported favourable effect of PG on rooting of different apple cultivars (James and Thurbon, 1979, 1981; Mehra and Saroj, 1979; Singha, 1982 and James, 1983). James and Thurbon (1979) reported auxin synergistic effect of PG in the process. The data scored in the present findings also revealed that direct rhizogenesis took place in presence of IBA (2.5, 5µM) + PG (10µM). In contrast to this Snir and Erez (1980) and Welender (1983) reported that PG (1mM) inhibited rooting in apple root stock A_2.

Use of shoot apices as explants for clonal micropropagation of apple has been found to be effective by Jones (1967), Pieniazek (1968), Elliot (1972), Powel (1970) Abbott and Whitely (1976), Zimmerman (1984) and Kumar and Kumar (1998). Present work also showed shoot tip explants to be highly effective in raising clonal (true to type) plants by direct regeneration. The presumption that more than 80,000 plants could be produced from single shoot tip in six months (Jones, 1967), can perhaps hold true after the refinement in presently established protocol, as very high number of direct multiple shoots were observed at basal end of subcultured shoots and the number continued to increase further after each subculture. Individual plantlets recovered in the present trials were looking healthy.

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