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Impact of BA and TDZ on *In Vitro* Shoot Proliferation of Two Indigenous Cultivars (Maharaji and Chambura) of Apple (*Malus Pumila* Mill.) in Kashmir

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ABSTRACT

Shoot apices of Maharaji and Chambura cultivars of apple (*Malus pumila),* obtained from young twigs of 40-50 year mature trees, were cultured on MS (1/2 strength) (1962) nutrient medium to assess the influence of different concentrations (0.5-5.0µM) of BA and TDZ separately and along with phloroglucinol(10µM) for multiple shoot formation. The explants of both the cultivars showed best response to BA (4.5µM) + PG (10µM) for shoot proliferation which was observed by direct multiple adventitious shoot regeneration at the base of the explant. The shoot lumps were subcultured 6-10 times, at an interval of 4 – 6 weeks, to increase the number of regenerated shoots. Each isolated shoot was then subcultured under the influence of a number of root inducing hormones. The best rooting response was scored on MS (½) +IBA (2.5µM) in both the cultivars. The plantlets thus obtained were transferred to pots containing peat vermiculite mixture (1:1) for hardening under laboratory conditions where survival rate was found to be 82% in Maharaji and 7% in Chambura.

Key words: *In vitro*, Maharaji, Chambura, maturetrees, shootapices, multipleadventitious 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, TDZ- Thidiazuron.

INTRODUCTION

Apple is a rosaceous fruit tree belonging to genus *Malus*. It is propagated in temperate regions of both northern and southern hemispheres of the world for its high economic value. The genus has five sections including 122 species and subspecies (Chadha and Awasthi, 2005). Over 700 accessions introduced from different parts of the world have been tried and tested in India from 1950 (Gosh, 2006). Natural varieties of cultivated apple belong to *Malus pumila* Mill. while its hybrid varieties belong to *Malus domestica* Bork. (Janick, 1996).

Nearly half of the production of apple is consumed as fresh fruit and most of the remainder is processed into apple juice, canned apple sauce, apple jam and apple butter. Dehydrated apples, apple flour, apple pie, apple dumpling, charoset (apple relish), apple haystacks are its other important commercial products. The fruit contains appreciable quantity sorbitol, and sugars (sucrose, glucose and fructose), organic acids (mainly malic and caproic acids) and vitamins. From medicinal view point, apple murraba, popular in India is regarded as a stimulant for heart. Fresh apple acts as purgative, prevents constipation, reduces incidence of dental caries, helps to control obesity and supplies extra energy for heavy exercise (Mitra, 1991). The pulp of apple fruit has been found to be the second richest source of phytochemicals like quercetin, catechin, phloridzin and chlorogenic acid, all of which are very strong antioxidants and reduce the risk of some cancers, cardiovascular diseases, asthma, and diabetes, inhibit cancer cell proliferation, decrease lipid oxidation, and lower cholesterol. Thus these prevent oxidative stress and delay ageing (Boyer and Liu, 2004).

Apple is propagated in all 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 10.38 metric tonnes (Anonymous, 2008). About 330 cultivars of apple were under cultivation in Kashmir Valley around 1978, but only few cultivars are seen at present in proliferating orchards. Because of poor returns, growers have stopped propagation of low yielding and less resistant varieties. This has led to drastic decline in the production of Maharaji, Chambura and other indigenous cultivars of apple and thus leaving their existence under threat.

Traditional method of propagation for apple is highly laborious, skilful and involves a lot of cost and wastage of time. Therefore, tissue culture technique seems to be the more reliable method for the production of self rooted clonal trees as it has the potential to provide large number of plants in less time. Although some *in vitro* work has been reported on other cultivars of apple from J & K state (Rizvi, 1999, Sharma *et al*., 2004, Dalal *et al.,* 2006, Rizvi *et al.,* 2007) but no work has been done till date on Maharaji and Chambura cultivars.

Present work thus represents the first report on *in vitro* culture of Maharaji and Chambura cultivars which are receiving less attention in the valley. The work will form a plateform for the conservation of these cultivars.

MATERIAL AND METHODS

Shoot apices of Maharaji and Chambura cultivars obtained from young twigs of mature fruit bearing trees (40-50 year old), growing in different orchards of Kashmir valley, were thoroughly washed under tap water using labolene (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. The explants were then were placed in Kn (15µM) solution in flasks for 24 hours at 4°C (in refrigerator) with their mouths sealed for avoiding phenolic exudation, after which they were re-sterilized using $HgCl_2$ (0.1%) for 90 seconds, followed by rinsing with autoclaved water several times to remove the traces of $HgCl_2$. The explants were then trimmed to 0.5-0.8cm long tips for inoculation on MS nutrient medium supplemented with different concentrations (0.5-5.0µM) of BA and TDZ either separately or with PG(10µM). The cultures were placed in incubation room where temperature was maintained between 22-28°C with light intensity of 3000 lux maintained for 18 hours daily.

Initially, the explants were transferred onto fresh nutrient media regularly after every 24 hours at least 5-7 times, for controlling browning of the medium but later on the culture products obtained were subcultured after every 4-6 weeks. The complete plantlets obtained were finally transferred from culture vials to small thumb pots for hardening.

RESULTS

Soot proliferation

Shoot apices (0.5- 0.8cm long) cultured under the influence of various BA and TDZ concentrations (0.5-5.0µM) separately or in combinations with PG(10µM) showed different degrees of response (Table-01). Best response in terms of shoot multiplication and proliferation was scored when medium was supplemented with BA (4-5µM) + PG (10µM) in both the cultivars tried. Phenolic exudations leading to browning of the medium and death of explants was avoided by chilling sterilized shoot tips in kinetin solution (15µM) over night, regular transfer of explants on fresh medium of same composition for 5-7 days and using the reduced (half) strength of MS salts. Culture establishment and shoot induction started after second week in Maharaji and third week in Chambura cultivar (Fig-1a & b). This was followed by direct multiple adventitious shoot

regeneration at the base of the explants (Fig-2a & b). The adventitious shoots thus produced were subcultured in lumps (6-10 times) after regular intervals of 4 – 6 weeks on same medium which continued to prolifer and increased shoot number by hundred folds (Table-01) (Fig-3a & b). During this period microshoots showed elongation as well. Both the selected cultivars responded best to BA (4.5µM) + PG (10µM) in terms of multiple shoot production. The average number of shoots produced per explant per subculture was 52±0.81 in Maharaji and 46±0.72 in Chambura.

Rooting of micro-shoots

Elongated micro-shoots were separated carefully from the lumps and subcultured on different rooting media where they showed varied response (Table-02). Best response was observed on MS (1/2) fortified with IBA (2.5µM) and PG (10µM) where 100 percent direct rhizogenesis was recorded in both the selected cultivars and average number of roots produced per shoot was 17±0.68 and 10±0.81 in Maharaji and Chambura respectively. When the concentration of IBA was increased to 5µM, percentage of response got drastically reduced to 51 and 36 in Maharaji and Chambura respectively. With IBA (2.5µM) root initials were seen in 2nd or 3rd week of subculture and profuse rooting was observed after six weeks of culture period (Fig-4a & b). The average number of roots produced per shoot was found to be 35±0.66 in Maharaji and 30±0.82 in Chambura with percent response of 80 and 70 respectively. Adventitious roots were also favoured by IBA (2.0µM) but percent response and mean root number were much lower than what was recorded with IBA (2.5µM). IBA (3.0-5.0µM) with PG (10 µM) initiated indirect roots. Rest of the rooting trials did not favour direct rooting but resulted in callusing at cut end (Table 02)

Complete plantlets worth transplantation were recovered in 8-10 weeks of rooting period. Roots started callusing soon after their initiation, when shoots were subcultured under the influence of triple auxin combination (IAA + IBA + NAA 1-5µM each) with or without PG (10µM). Rooted shoots (4-8cm long) were later on properly deflasked as per its established protocol and transferred to pots containing sand- soil mixture of 1:1 for acclimatization under high humidity under lab conditions (Fig-5a & d). The percentage of survival was observed to be 82% in Maharaji and 78% in Chambura cultivar.

Table 1. Impact of different concentrations of BA and TDZ used either separately or with PG(10µM) on the shoot apices from mature trees of different cultivars of apple cultured *in vitro* on MS (half-strength) nutrient medium.

Phytohormones (µM)	Nature of Response		Percentage of response		Shoot Number Mean±SD	
	MJ	CH	MJ	CH	MJ	CH
CONTROL	NR	NR	0	0	NA	NA
BA (0.5)	NR	NR	0	0	NA	NA
BA (1.0)	NR	NR	0	0	NA	NA
BA (1.5)	NR	NR	0	0	NA	NA
BA (2.0)	CCE	CCE	5	2	NA	NA
BA (2.5)	CCE	CCE	6	2	NA	NA
BA (3.0)	CCE	CCE	4	3.6	NA	NA
BA (3.5)	CCE	CCE	15	4	NA	NA
BA (4.0)	ASP	ASP	18	14	14±0.88	22±0.82
BA (4.5)	ASP	ASP	18	15	23±0.88	14±0.82
BA (5.0)	ASP	ASP	20	16	26±0.82	14±0.72
BA (0.5) + PG (10)	NR	NR	0	0	NA	NA
BA (1.0) + PG (10)	NR	NR	0	0	NA	NA
BA (1.5) + PG (10)	NR	NR	0	0	NA	NA
BA (2.0) + PG (10)	NR	NR	0	0	NA	NA
BA (2.5) + PG (10)	CCE	CCE	27	18	NA	NA
BA (3.0) + PG (10)	CCE	CCE	28	20	NA	NA
BA (3.5) + PG (10)	CCE	CCE	28	21	NA	NA

Table 1 Contd..

Table 1 Contd..

BA (4.0) + PG (10)	ASP	ASP	92	80	48±0.82	42±0.71
BA (4.5) + PG (10)	ASP	ASP	90	75	52±0.81	46±0.72
BA (5.0) + PG (10)	ASP	ASP	90	72	52±0.76	46.±0.82
TDZ (0.5)	NR	NR	0	0	NA	NA
TDZ (1.0)	NR	NR	0	0	NA	NA
TDZ (1.5)	NR	NR	0	0	NA	NA
TDZ (2.0)	CCE	CCE	17	12.5	NA	NA
TDZ (2.5)	CCE	CCE	15	12.6	NA	NA
TDZ (3.0)	CCE	CCE	16	12.9	NA	NA
TDZ (3.5)	ASP	ASP	22.8	15.0	12±0.76	10±0.71
TDZ (4.0)	ASP	ASP	29	27	18±0.82	15±0.71
TDZ (4.5)	ASP	ASP	30	27	28±0.72	16±0.72
TDZ (5.0)	ASP	ASP	29	25	27±0.76	16.±0.82
TDZ (0.5) + PG (10)	NR	NR	0	0	NA	NA
TDZ (1.0) + PG (10)	NR	NR	0	0	NA	NA
TDZ (1.5) + PG (10)	NR	NR	0	0	NA	NA
TDZ (2.0) + PG (10)	NR	NR	0	0	NA	NA
TDZ (2.5) + PG (10)	NR	NR	0	0	NA	NA
TDZ (3.0) + PG (10)	CCE	CCE	25	12	NA	NA
TDZ (3.5) + PG (10)	CCE	CCE	28	10	NA	NA
TDZ (4.0) + PG (10)	CCE	CCE	32	12	NA	NA
TDZ (4.5) + PG (10)	CCE	CCE	32	27	NA	NA
TDZ (5.0) + PG (10)	CCE	CCE	35	27	NA	NA

MJ- Maharaji, CH – Chambura. CCE - Callus at Cut End, ASP – Adventitious Shoot Proliferation
NR - No Response, NA- Not Applicable; Ten replicates/ treatment

Data scored after every six weeks and representing mean of ten subcultures.

Table 2. *In vitro* response of sub-cultured shoots of different cultivars of apple to rooting hormones used in MS (half-strength) nutrient medium.

Phytohormones (µM)	Nature of Response		Percentage of response		Root Number Mean±SD	
	MJ	CH	MJ	CH	MJ	CH
CONTROL	NR	NR	NR	NR	NR	NR
IBA (0.5)	NR	NR	0	0	NA	NA
IBA (1.0)	CCE	CCE	0	0	NA	NA
IBA (1.5)	CCE	CCE	0	0	NA	NA
IBA (2.0)	ARF	ARF	32	21	12±0.85	13±0.88
IBA (2.5)	ARF	ARF	80	74	35±0.66	30±0.82
IBA (3.0)	CR	CR	52	38	15±0.80	13±0.82
IBA (3.5)	CR	CR	15	4	14±0.74	12±0.71
IBA (4.0)	CR	CR	15	4	15±0.81	11±0.82
IBA (4.5)	CR	CR	15	4	18±0.88	23±0.81
IBA (5.0)	CR	CR	15	4	26±0.82	24±0.72
IBA (0.5) + PG (10)	NR	NR	0	0	NA	NA
IBA (1.0) + PG (10)	NR	NR	0	0	NA	NA
IBA (1.5) + PG (10)	NR	NR	0	0	NA	NA
IBA (2.0) + PG (10)	CR	CR	52	37	25±0.82	13±0.81
IBA (2.5) + PG (10)	ARF	ARF	100	100	17±0.68	10±0.81
IBA (3.0) + PG (10)	CR	CR	72	68	18±0.65	19±0.82
IBA (3.5) + PG (10)	CR	CR	70	62	23±0.65	18±0.81
IBA (4.0) + PG (10)	CR	CR	72	62	33±0.65	28±0.81
IBA (4.5) + PG (10)	CR	CR	72	65	32±0.68	28±0.73
IBA (5.0) + PG (10)	CR	CR	51	36	28±0.67	24±0.77
IAA (0.5)	NR	NR	0	0	NA	NA
IAA (1.0)	NR	NR	0	0	NA	NA

Table 2 Contd…

Table 2 Contd…

IAA (1.5)	NR	NR	0	0	NA	NA
IAA (2.0)	NR	NR	0	0	NA	NA
IAA (2.5)	NR	NR	0	0	NA	NA
IAA (3.0)	NR	NR	0	0	NA	NA
IAA (3.5)	NR	NR	0	0	NA	NA
IAA (4.0)	CCE	CCE	32	31	NA	NA
IAA (4.5)	CCE	CCE	32	32	NA	NA
IAA (5.0)	CCE	CCE	28	27	NA	NA
NAA (0.5)	NR	NR	0	0	NA	NA
NAA (1.0)	NR	NR	0	0	NA	NA
NAA (1.5)	NR	NR	0	0	NA	NA
NAA (2.0)	NR	NR	0	0	NA	NA
NAA (2.5)	NR	NR	0	0	NA	NA
NAA (3.0)	CCE	CCE	10	10	NA	NA
NAA (3.5)	CCE	CCE	20	22	NA	NA
NAA (4.0)	CCE	CCE	32	35	NA	NA
NAA (4.5)	CCE	CCE	28	25	NA	NA
NAA (5.0)	CCE	CCE	27	28	NA	NA

MJ- Maharaji, CH - Chambura,

CCE - Callus at Cut End, ARF– Adventitious Root Formation, CR – Callose Roots

NR - No Response, NA- Not Applicable, Ten replicates/treatment

Data scored after six weeks.

DISCUSSION

Present investigation was carried out chiefly to explore organogenetic potential of shoot apices taken from mature trees of Ambri, Chambura, Maharaji, Golden Delicious apple cultivars to obtain clonal plantlets. The key factors governing cloning of apple through *in vitro* means were observed to be proper sterilization, pre-inoculation chilling, control over oxidative browning, strength of nutrient medium, appropriate hormonal concentration andhardening.

Fig-1a

Fig-1b

Fig-2a

Fig-2b

Fig-3a

Fig-3b

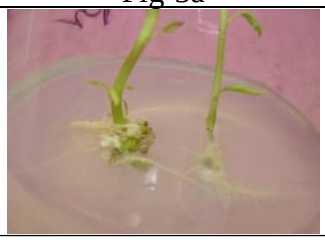

Fig-4a

Fig-4b

Fig-5a

Fig-5b

Figs 1-5: Morphogenetic response of shoot apices from mature trees of Maharaji and Chambura cultivars of apple (Malus pumila) to various phytohormones

Legend:

Fig-1a: Shoot induction in Maharaji cultivar from mature shoot apex on MS (1/2) + BA (5µM) + PG (10µM) (after 2nd week)

Fig-1b: Shoot induction in Chambura cultivar from mature shoot apex on MS (1/2) + BA (5µM) + PG (10µM) (after 3rd week)

Fig-2a: Direct multiple adventitious shoot formation in Maharaji cultivar on MS (1/2) + BA (5µM) + PG (10µM) (after 5th week)

Fig-2b: Direct multiple adventitious shoot formation in Chambura cultivar on MS (1/2) + BA (5µM) + PG (10µM) (after 5th week)

Fig-3a: Subcultured multiple shoots of Maharaji cultivar on MS (1/2) + BA (5µM) + PG (10µM) (after eight weeks)

Fig-3b Subcultured multiple shoots of Chambura cultivar on MS (1/2) + BA (5µM) + PG (10µM) (after eight weeks)

Fig-4a: Rooting of subcultured shoots of Maharaji cultivar on MS (1/2) + IBA (2.5µM) + PG (10µM) (after six weeks of subculture)

Fig-4b: Rooting of subcultured shoots of Chambura cultivar on MS (1/2) + IBA (2.5µM) + PG (10µM) (after six weeks of subculture)

Fig-5a: Deflasked plantlet of Maharaji cultivar in Sand-soil mixture 1:1 for acclimatization.

Fig-5b: Deflasked plantlet of Chambura cultivar in Sand-soil mixture 1:1 for acclimatization.

In present work, initial treatment of explants 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 for successful establishment of explants in all the cultivars under study. In contrast to this Zimmerman (1984) suggested use of calcium hypochlorite (20%) for controlling microbial contamination in apple cultivars.

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°C in Kn (15μM) after initial sterilization, regular transfer (5-7 times) onto fresh nutrient medium of same composition and reduction of MS salt strength to half have been found to be effective steps for controlling browning.

Different nutrient media have been tried from 1958 onwards for establishment and proliferation of adventitious multiple shoots from shoot apices different cultivars of apple, like Knop's salt solution by Jones (1976), W-63 by Saad (1965), MiS by Messer and Lavee (1969), DP by Powel (1970), EL by Elliott (1972), FN by Fuji and Nito (1972), K(Knudson) by Jones and Hatfield (1976), QM by Snir and Erez (1980), LS by James and Thurbon (1981), 8P by Niizeki *et al.,* (1983), Nitsch's medium by Koudir *et al.* (1984), Lapovior medium by Le (1985), Gamborg's medium by Barberi and Moorini (1987), KSMP by Doughty and Power (1988), WPM by Orlikowska (1988), DKW by Wilson and James (2003). Most other persons like Zimmerman (1984), Anderson (1990), Dong *et al.* (1995), Caboni *et al.* (2000), Hoffmann *et al.,* (2001), Lambert & Tepfer (2001), Hofmann *et al.* (2001), Martins *et al* (2001), Sicurani *et al* (2001). Dobránszki *et al.,* (2002), Kadota *et al.* (2002), Cheng *et al.* (2003), Damiano and Monticelli (2003), Hao and Deng (2003), Hofer (2004), Sharma *et al.* (2004), Modgil *et al.* (2005), Allan *et al.* (2006), Dalal *et al.* (2006), Dandekar *et al.* (2006), Dantas *et al.* (2006), Goani *et al.* (2006), Bisogenin *et al.* (2008) have used MS (1962). In present experiment MS medium was tried which gave favourable results.

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.

Shoot proliferation leading to multiple shoot proliferation was very much recorded in present work by using PG (10µM) and BA (4-5µM) in MS (1/2) medium. Earlier proliferation of shoots of M.7 and M.26 apple cultivars 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) which supports our results. Sharma *et al.* (2004) have found TDZ more effective than BA on Ambri cultivar which contradicts our findings on Maharaji and Chambura cultivars. Recently Dalal *et al.* (2006) have reported BA (2.22 µ*M*) effective for shoot proliferation which is in corroboration with our results.

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); Correa *et al.* (1990); Nui *et al.* (1995) and Puntae and Martin (1997) but contradicts the findings by Dalal *et al* (2006) who have used very low concentration of IBA (0.49µ*M*) instead of 2.5µ*M* .

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 other workers have also 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. 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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