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In-vitro Regeneration of Apple Cultivar (*Malus domestica* L. cv. Galmast)

Subzar Ahmad Rather* and Junaid Jalal

Department of Biotechnology, Mewar University, Gangarar, Chitttorgarh (Rajasthan), India
**Corresponding author*

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A B S T R A C T

Double sterilized and pre-chilled shoot apices of Galmast cultivar obtained from mature trees (MTSTs) and in vitro raised seedlings (SBSTs) were inoculated on MS medium augmented with BA, Kn and TDZ with and without PG. Increase in BA concentration up to 5.0µM resulted in axillary shoot proliferation in about 40% shoots with a maximum of 14±0.72 shoots per explant. SBSTs (seedling born shoot tips) of same cultivar developed light yellow loose callus (LYLC) when they were cultured on MS(×½) medium fortified with BA(2.0-3.5µM) and produced axillary and adventitious shoots when the same medium was supplemented with BA(4.0-5.0µM. The best response was observed on MS(1/2) + BA(4µM) + PG(10µM) However, about 40% SBSTs showed axillary shoot proliferation on MS(1/2) + TDZ (4µM) + PG(10µM) and produced a maximum average number of 40±0.72 shoots per subculture. However, 25-30% shoots showed axillary shoot proliferation (ASP) with further increase in the concentration of TDZ to 4.0, 4.5 and 5.0µM with PG (10µM). The number of shoots produced was 14±2.11, 12±0.32 and 14.±0.45. The shoots obtained through the mature tree explant culture (MTEC) and seedling born explant culture (SBEC) were sub cultured onto rooting medium containing auxins like IBA, IAA, NAA. Increase in its concentration to 2.0 and 2.5µM resulted in development of adventitious roots in about 60% plants obtained through MTEC with an average of 14±0.82 roots per shoot. When the medium was fortified with IBA(2.0µM) + PG(10µM). When the concentration of IBA was increased further to 2.5µM the micro shoots produced adventitious roots (with cent per cent response) having an average number of 8±0.65 roots per micro shoot. Further increase in the concentration of IBA to 3.0, 3.5, 4.0, 4.5and 5.0µM favoured formation of callose roots but with decreased response. Best root initiation and elongation to obtain complete plantlets from the shoots derived from explants was observed on MS (1/2) supplemented with IBA(2.5µM) + PG(10µM).

Introduction

Propagation of woody plants by conventional methods necessarily limits the rate of output and makes the end product expensive. Tissue culture can overcome this problem since it has been reported that may acquire higher rooting capability after continuously subculturing in vitro (Hammatt & Grant., 1993). Tissue culture techniques such as micropropagation provide a fast and dependable method for the production of a large quantity of uniform

plantlets in a short time throughout the year (Zimmerman., 1981). Recently in apple, many reliable methods have been developed for both propagation of rootstocks and scions using in vitro techniques. Successful micropropagation of apple using microcuttings or single node cuttings is influenced by several internal and external factors, including genotype, physiological state of sampling, in vitro media constituents and their ratio, light, temperature and other factors(Dobranski *et al.*, 2002). Apple (*Malus* × *domestica* Borkh.;*Rosaceae*) is an important fruit crop grown mainly in temperate regions of the world. In vitro tissue culture is a biotechnological technique that has been used to genetically improve cultivars (scions) and rootstocks. This updated review presents a synthesis of findings related to the tissue culture of apple and other *Malus* spp. between 2010 and 2018. Increasingly complex molecular studies that are examining the apple genome, for example, in a bid to identify the cause of epigenetic mutations and the role of transposable elements in this process would benefit from genetically stable source material, which can be produced in vitro. Several notable or curious in vitro culture methods have been reported to improve shoot regeneration and induce the production of tetraploids in apple cultivars and rootstocks. Existing studies have revealed the molecular mechanism underlying the inhibition of adventitious roots by cytokinin. The use of the plant growth correction factor allows hypothetical shoot production from leaf-derived thin cell layers relative to conventional leaf explants to be determined. This updated review will allow novices and established researchers to advance apple and *Malus* biotechnology and breeding programs.

The tissue culture of domesticated apple (*Malus* × *domestica*Borkh.) has a rich and extensive history spanning approximately 60 years (Dobránszki and Teixeira da Silva., 2015). Since the apple genome is highly heterozygous, a consistent genetic background in a given cultivar can be maintained only by vegetative propagation, i.e., cloning. This would be important in the production of genetically uniform scions and rootstocks for commercial apple production. In nurseries, apple plants are produced by grafting scions onto rootstocks. The rootstock determines some important traits of grafted trees, including growth vigour, yield and resistance or tolerance to biotic and abiotic stresses (Rom and Carlson.,1987; Laimer M.,, Barba M., 2011). The Cornell-Geneva (Geneva® series) breeding program has bred several dwarf rootstocks that are resistant to diseases and pests and are also cold hardy.[1] Several of these rootstocks have been extensively researched in recent years*2,4-D* 2,4-dichlorophenoxyacetic acid, *2iP* 6-(γ,γ-dimethylallylamino) purine, *AA* ascorbic acid; *ABA*abscisic acid, *AC* activated charcoal, *AD* apical dome, *Alar-85*daminozide, *APM* amiprophos methyl, *ASGV* apple stem grooving virus, *ASPV* apple stem pitting virus, *B5* medium (Gamborg *et al.*,1968), *BAN*^{6}-benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original (Teixeira da Silva.,2012a), *BAR* N^6-benzyladenine riboside, *CA* citric acid, *CH* casein hydrolysate, *CIM* callus induction medium; *d* day(s), *DKW* Driver and (Kuniyuki., 1984) medium, *GA*$_3$gibberellic acid, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid; *ISSR* inter simple sequence repeat, *Kin* kinetin (6-furfuryl aminopurine); *LP* leaf primordium; *mo* month(s); *MS*(Murashige and Skoog., 1962) medium, *mT meta*-topolin (6-(3-hydroxybenzylamino)purine) or *N*^{6}-meta-hydroxy-benzyladenine, *mTR meta*topolin-riboside or *N*^{6}-metahydroxy-benzyladenine ribozide, *NAA* α-naphthalene-acetic acid, *NR* not reported in the study, *PG*phloroglucinol, *PGCF* plant growth correction factor (Teixeira da Silva and Dobránszki., 2011, 2014), *PGR* plant growth regulator, *PGPR* plant growth-promoting rhizobacteria, *PIC*picloram (4-

amino-3,5,6-trichloro-pyridine-2-carboxylic acid), *PP* photoperiod, *PP333*paclobutrazol, *PPFD* photosynthetic flux density, *PVP* polyvinyl pyrrolidone, *QL* (Quoirin and Lepoivre) macroelements, Quoirin and (Lepoivre., 1977), *RAPD* random amplified polymorphic DNA; *REM* root elongation medium, *RIM* root induction medium, *ROS* reactive oxygen species, *SEM* shoot elongation medium, *SIM* shoot induction medium, *SMM* shoot multiplication medium, *SN* shoot number (or number of shoots), *SSR* simple sequence repeat, *TDZ* thidiazuron (*N*-phenyl-*N*'- 1,2,3-thiadiazol-5-ylurea), *tTCL* transverse thin cell layer, *w* week(s), *WPM* woody plant medium (Lloyd and McCown., 1980).

About 330 varieties of apple are known to have been under cultivation in Kashmir valley around 1972 but only a dozen are propagated at present on commercial scale. J & K state has remained popular for its indigenous apple varieties.Galmast striped red and yellow, this New Zealand native was brought to the United States in the early 1970s. Crisp, juicy and very sweet, Gala is excellent for snacks or salads, and is also good for pies, sauce and baking. U.S. Galas are available nationwide year-round.

Gala apples bring a sweet, clean crunch to the table. This apple is one of the first apples of the season to ripen, brightening up green summer foliage with hues of red over yellow. Most Gala apple trees (like Starkrimson® Gala) produce small to medium fruit. For a larger piece of the "apple pie", choose the Stark® GrandGala™. These trees are excellent pollination companions for any Golden Delicious variety; and with both a Golden and a Gala growing in your yard, you'll be all set for some blue ribbon pies!This striped yellow and red apple is sweet and firm. Excellent for snacking and salads, Galmast is also a good for pies, baking and sauce making. U.S. Galmast are available nationwide year-round. The present study aims for developed of efficient plant regeneration protocol of apple (cv. Galmast) from shoot apices.

Materials and Methods

The present research work was carried out with the aim assessing morphogenetic and organogenetic potential of explants from mature trees and in vitro raised seedlings to develop complete protocol for mass propagation of apple cultivar i.e. Galmast

Collection of Explants

The source material was obtained from mature trees and in vitro born seedlings. The explants used from mature trees were obtained from different orchards at Zakura, Shopian and Pattan area in District Baramulla. The explants used as experimental material were 2-3cm long sprouts which were cut by sterile razors and collected in polythene bags containing moist cotton to prevent wilting and were taken to laboratory. Explants were either processed for inoculations immediately or placed in a refrigerator overnight. In vitro born seedlings / shoots obtained after aseptic germination of mature apple seeds.

Sterilization of Plant Material

Shoot apices (0.5 cm long), obtained from young and actively growing shoots of 40-50 year old mature trees were placed in enamel trays containing tap water with two to three drops of detergent (Labolene 5%) and a drop Tween-20 (surfactant).

The explants were stirred gently and then washed with running tap water until all the traces of soap were completely removed. The explants were then placed in different sterilants for different time durations to attain complete asepsis and then rinsed 3 times with filtered water (obtained from water purifier) and finally with double distilled water. They

were then kept in 10-25µM kinetin solution overnight (24hours) in refrigerator at 4^{O}C to reduce leaching of phenolic compounds. Next day further processes were carried under Laminar air flow cabinet.

Table.1 Composition of different media used for apple tissue culture

S.No	Medium	WM	MSM	QM	WPM
		White (1943)	Murashige and Skoog(1962)	Quoirin(1972)	Lloyd and Mc. Crown (1980)
	Concentration→ Ingredients ↓	mg/L	mg/L	mM	mg/L
A	**Macronutrients**				
01	NH_4NO_3	-	1650	5.0	400
02	KNO_3	80	1900	17.8	-
03	$CaCl_2.2H_2O$	-	440	-	96
04	$MgSO_4.7H_2O$	750	370	1.5	370
05	K_2SO_4	-	-	-	990
06	KH_2PO_4	-	170	2.0	170
07	$(NH_4)_2SO_4$	-	-	-	556
08	$Ca(NO_3)_2.4H_2O$	300	-	5.1	-
09	Na_2SO_4	200	-	-	-
10	$NaH_2PO_4.H_2O$	19	-	-	-
B	**Micronutrients**				
01	KI	0.75	0.83	0.1	-
02	KCl	65	-		
03	H_3BO_3	1.5	6.2	0.1	6.2
04	$MnSO_4.H_2O$	5.0	22.3	0.1	22.3
05	$MnSO_4.4H_2O$	-	-	-	0.1
06	$ZnSO_4.7H_2O$	3.0	8.6	0.03	8.6
07	$CuSO_4.5H_2O$	0.01	0.025	0.0001	0.25
08	$Fe_2(SO_4)_3$	2.5	-	-	-
09	MoO_3	0.001	-	-	-
10	$CoCl_2.6H_2O$	-	0.025	0.0001	-
11	$Na_2MoO_4.2H_2O$	-	0.25	0.001	0.25
12	$Fe_2(SO_4)_3.7H_2O$	-	27.8	0.1	27.8
13	$Na_2EDTA.2H_2O$	-	37.3	0.1	37.3
C	**Organics**				
01	Thiamine HCl	0.01	0.1	0.0012	0.25
02	Nicotinic acid	0.01	0.5	0.0041	-
03	PyridoxinHCl	0.01	0.5	0.0024	-
04	Glycine	3.0	2.0	-	0.5
05	Myo-inositol	-	100	0.56	100
06	Sucrose	2.0%	3.0%	2.0%	2.0%
07	Agar	0.8%	0.8%	0.8%	0.8%

Table.2 Stock solutions of the different constituents of MS (1962) medium

Stock Solution Code (MSSS-01)			**Major Salts [strength =10X]**	
Constituents	Quantity (mg/L) in original medium	Quantity dissolved in stock solution	Final volume of the stock solution	Qua. to be used for preparation of one Litre MS Medium
NH_4NO_3	1650	16.5g	1000 ml	100 ml
KNO_3	1900	19.0g		
$CaCl_2.2H_2O$	440	04.4g		
$MgSO_4.7H_2O$	370	03.7g		
KH_2PO_4	170	01.7g		
Stock Solution Code (MSSS-02)			**Minor Salts [strength =100X]**	
KI	0.83	08.3g	500ml	5.0ml
H_2BO_3	6.20	62.0g		
$MnSO_4.4H_2O$	22.30	2230g		
$ZnSO_4.7H_2O$	8.60	8.60g		
$Na_2MoO_4.2H_2O$	0.250	0.25g		
$CuSO_4.5H_2O$	0.025	0.025g		
$CoCl_2.6H_2O$	0.025	0.025g		
Stock Solution Code (MSSS-03)			**Iron Source [strength =100X]**	
$FeSO_4.7H_2O$	27.8	2.78g	500ml	5.0ml
Na_2 EDTA.$2H_2O$	37.3	3.73g		
Stock Solution Code (MSSS-04)		**Myo-Inostol [strength =50X]**		
Myo-inositol	100	5.0g	250ml	5.0ml
Stock Solution Code (MSSS-05)		**Organic ingredients [strength =100X]**		
Thiamine HCl (B_1)	0.1	10.0mg	500ml	5.0ml
Nicotinic acid (B_5)	0.5	50.0mg		
Pyridoxine HCl (B_6)	0.5	50.0mg		
Glycine	2.0	200.0mg		

Selection of Nutrient Media

Four nutrient formulations namely (White's., 1943) medium, (Murashige and Skoog's (MS) (1962) medium, (Quoirin *et al.*, 1977) but the explants responded well on MS medium. Thus all trials were later on carried on MS (1962) medium. However, use of full strength salt formulations with media supplements yielded

poor results to the reduced salt strength i.e. half salt strength. The medium was supplemented with different auxins (2,4-D, IAA, NAA and IBA), cytokinins (BA and Kn), gibberellin (GA3) and PG and TDZ in different concentrations and combinations and encouraging results were yielded.

Preparation of Stock Solutions

Weighing of all constituents of a nutrient medium individually and their mixing was made to the highest level of accuracy. Concentrated stock solutions of major salts, minor salts, myoinositol, iron source, vitamins and phytohormones were prepared on need basis which not only saved time but was more accurate. The stock solutions were kept in dark glass bottles and stored in refrigerators. The strength and composition of stock solutions is depicted in Table 2.

Preparation of Nutrient Medium

For all trials the medium was prepared in sterile vials of Borosil glass. Required quantities from (pre-prepared) stock solutions of MS major and minor salts, vitamins and myo-inositol were mixed together for one litre basal medium. This was followed by the addition of phytohormones from their stock solutions as per the need. (Table 5). Double distilled water was added to increase the volume of the solution. Sucrose (3%) was added and allowed to get dissolved properly. pH of the solution was adjusted between 5.2-5.8 by adding NaOH (0.1N) or HCl (0.1N) drop by drop. Final volume of the medium was adjusted, by adding more double distilled water, before the medium was gelled with 0.8 % agar. The medium was finally dispensed in different culture vials which were then tightly plugged with sterilized cotton. It was then autoclaved at 15–20 pounds/inch pressure at 121^{o}C for 20 minutes and then allowed to cool.

Inoculation

Sterilized explants were inoculated onto aseptic basal medium (control) and phytohormone supplemented medium on the hood of Laminar air flow chamber. The culture vials were then placed in incubation room under cool fluorescent illumination.

Subculture

Subculture was carried out on the hood of Laminar air flow chamber under aseptic conditions after every 4-6 weeks depending upon the organogenetic and proliferation potential of the explants. The products of explants were carefully separated out and inoculated in separate vials.

Regular Observation and Data Recording

The cultures were daily monitored for contamination and growth. The changes in explant were recorded on weekly basis and the data was put in proper sequence and in tabulated form. It was also transcribed at the end of every week and stored as e-content.

Data Analysis and Interpretation

After recording correct and accurate data about nature of media used, phytohormonal concentration, date of inoculation, incubation and subculture, nature of light i.e., its intensity, quality and duration, humidity etc. and their impact on explant response, callus growth, organogenesis, embryogenesis, it was analysed through statistical and mathematical methods. Ten / twenty replicates were taken for each treatment and observations were recorded at the end of every week. Analysis of variance (ANOVA) was carried to determine the significance of the results using Dunkan's multiple range test ($a \leq 0.05$) for mean number of shoots/ roots produced.

Hardening

The plantlets obtained from different explants through repeated subcultures were finally left in culture vials with open mouth for three days in the incubation room, transferred to thumb pots containing peat-vermiculite or soil-peat mixture and then taken out of incubation room of the lab. Attempts were made to acclimatize plants under laboratory conditions as the green house facility could not be availed due to its late installation in the campus.

Results and Discussion

Culture Establishment and shoot multiplication

Double sterilized and pre-chilled shoot apices of Galmast cultivar obtained from mature trees (MTSTs) and *in vitro* raised seedlings (SBSTs) were inoculated on MS medium augmented with BA, Kn and TDZ with and without PG the results of which are summarised in tables 3 and 4. As in case of Red fuji and Golden Delicious cultivars, little response was seen on MS medium with full strength of its salts but good results were recorded when the strength of the medium was reduced to half.

Impact of BA alone

Under the influence of BA(0.5-1.5µM) the MTSTs (mature tree shoot tips) turned brown within 48 hours and then faced necrosis. Increase in BA concentration upto 3.5µM resulted in the formation of light yellow callus (LYC) at cut ends in about 35% shoo tips. Further increase in its concentration upto 5.0µM resulted in axillary shoot proliferation in about 40% shoots with a maximum of 14±0.72 shoots per explant (Table 3).

SBSTs (seedling born shoot tips) of same cultivar developed light yellow loose callus (LYLC) when they were cultured on MS(×½) medium fortified with BA(2.0-3.5µM) and produced axillary and adventitious shoots when the same medium was supplemented with BA(4.0-5.0µM). However, only a maximum of 70% cultures developed multiple shoots with maximum average number of 24±0.72 shoots per shoot per subculture (Table 4).

Impact of BA with PG

Culture of MTSTs on MS(1/2) supplemented with BA and PG yielded good results (Plate-A, Figs.01 and 02). No response was seen when they were cultured under the influence of low cytokinin i.e. BA(0.5-2.0µM) + PG(10µM). Increase in BA concentration from 2.0 to 3.5µM resulted in the development of callus at cut ends in 25% shoots. Further increase in BA concentration from 4.0 to 5.0µM with PG(10µM) favoured growth of apical bud as well as axillary buds and their proliferation (ASP). The best response was observed on MS(1/2) + BA(4µM) + PG(10µM) (Plate-B, Figs.03). Unlike Red fuji and Golden Delicious cultivars increase in BA concentration from 4.0 to 4.5 and 5.0µM decreased percentage of response from 90% to 70% and number of adventitious and axillary shoots from 42±0.71 to 36.±0.82 per shoot as depicted in table 3. The shoots obtained were subcultured individually or in lumps several times to increase number of shoots (Plate-B, Figs.04 and 05 and Plate-A, Fig.01). The potential of shoot proliferation continued in the subcultures attempted. SBSTs of same cultivar developed light yellow loose callus (LYLC) when the medium was fortified with BA(2.0-3.5µM)+ PG(10µM) but developed axillary and adventitious shoots when the medium was supplemented with BA(4.0-5.0µM)+ PG(10µM) (Plate-A, Figs.01) . Best (cent percent) response was seen on the medium containing BA(4.0µM) when a maximum average number of 50±1.35 shoots

were produced per shoot per subculture (Plate-B, Figs. 02 and 03) (Table 4). The potential of shoot proliferation increased in the subcultures attempted. Similar findings were reported by (Kouider *et al.,* 1986), whose work focused on micropropagation of quince. They showed that the rate of multiplication increases with the increase in the amount of BAP, while, the rate of elongation decreases. (Gladyshava., 1987) in trying to multiply the papaw tree in vitro, showed that the amount of BAP is positively correlated at the rate of multiplication.

Impact of TDZ alone

Use of TDZ did not yield successful results in case of MTSTs and only a maximum of 30% shoots produced loose creamy callus (LCC) at cut ends (CCE). However, about 40% SBSTs showed axillary shoot proliferation on MS(1/2) + TDZ(4µM) + PG(10µM) and produced a maximum average number of 40±0.72 shoots per subculture (Table 3).

Impact of TDZ with PG

Addition of PG to the medium containing TDZ did not help in getting successful results during the culture of MTSTs as no response was seen while using 0.5 2.5µM TDZ with PG(10µM). Loose creamy callus (LCC) at cut ends (CCE) was observed when concentration of TDZ was increased to 3.0 and 3.5 µM with PG(10µM). However, 25-30% shoots showed axillary shoot proliferation (ASP) with further increase in the concentration of TDZ to 4.0, 4.5 and 5.0µM with PG(10µM). The number of shoots produced was 14±2.11, 12±0.32 and 14.±0.45 respectively (Table 3).

When SBSTs of same cultivar were cultured under the influence of TDZ+PG callus formation was observed under low concentrations of TDZ (3.0 and 3.5µM) and axillary shoot proliferation was seen in 45% shoots (Table 4).

Previous studies on apple reveals that explants from in vitro raised seedlings have higher organogenetic potential when compared with those obtained from mature trees. Present findings run parallel to it whereby seedling based explants have shown three times greater organogenetic potential than adult tree based explants. This is, however, of little practical importance as plants raised from seedlings in field never yield fruits unless they are subjected to grafting. While working on shoot tips of different apple cultivars most researchers have reported shoot proliferation and direct multiple shoot formation under the influence of BA (1.0-5.0 µM) alone (Ciccotti *et al.,* 2008; Bahmani, *et al.,* 2016). In present work direct shoot multiplication in the shoot tips from mature trees was achieved when the medium was fortified with BA (4.0-5.0 µM) alone, BA(4.0-5.0 µM) + PG(10µM) and TDZ(4.0-5.0 µM) + PG(10µM) but best results were observed only under the influence of BA(4.0-5.0 µM) + PG(10µM).

Thus results achieved on the culture of shoot apices run parallel to the findings of (James and Thurbon., 1981 : Zimmerman and Broome., 1981 and Modgil *et al.,* 1994) but contradicts with the findings of other researchers. Some researchers like (Sharma *et al.,* 2004 : Nabeela *et al.,* 2009) have succeeded in inducing direct shoot multiplication in the shoot apices of some apple cultivars under the influence of TDZ and have reported BA to be less effective in comparison to TDZ. On the other hand, (Welander., 1988) have reported direct shoot multiplication when the medium was augmented with PG alone. In contrast to these findings, (Modgil *et al.,* 1994) have achieved great success the same by using BA (1-5.0µM) with PG.

Rooting of *in vitro* raised Shoots

The shoots obtained through the mature tree explant culture (MTEC) and seedling born explant culture (SBEC) were subcultured onto

rooting medium containing auxins like IBA, IAA, NAA as summarised in (tables 4 and 5).

Impact of IBA without PG

Use of 1.0 and 1.5µM IBA alone promoted callus formation at basal end in about 5% shoots. The callus produced was creamy yellow (CYC). Increase in its concentration to 2.0 and 2.5µM resulted in development of adventitious roots in about 60% plants obtained through MTEC with an average of 14±0.82 roots per shoot (Table 4).

The shoots obtained through SBEC also showed similar response. About 85% shoots produced adventitious roots with an average of 12±0.82 roots per shoot when the medium was fortified with IBA(2.5µM).

Impact of IBA with PG

Whereas callose roots were produced when the concentration of IBA was increased to 2.0µM. Further increase in the concentration of IBA to 2.5µM resulted in the initiation and development of adventitious roots in all shoots (table 5) (100% response) with an average of 12±0.81 roots per shoot (Plate-A, Fig.02; Plate-A, Fig.03; Plate-C- Fig.03).

Microshoots of Galmast apple cultivar obtained through SBEC cultured under the influence of IBA (0.5, 1.0 and 1.5µM) with PG(10µM) did not show any response and produced callose roots when the medium was fortified with IBA(2.0µM) + PG(10µM). When the concentration of IBA was increased further to 2.5µM the microshoots produced adventitious roots (with cent percent response) having an average number of 8±0.65 roots per microshoot. (Plate-A, Figs.04 and 05; Plate-A, Fig.06; Plate-A, Fig.03; Plate-C, Fig.05). Further increase in the concentration of IBA to 3.0, 3.5, 4.0, 4.5and 5.0µM favoured formation of callose roots but with decreased response.

Impact of IAA, NAA and IBA+IAA+NAA

When the microshoots obtained through MTEC were cultured under the influence of IAA or NAA no response was seen until their concentration was increased to 3.0µM while callus formation was observed at cut ends (CCE) of microshoots at 3.0-5.0µM concentration in about 30% shoots. The callus produced was creamy yellow and loose (CYC). Rooting could not be achieved with the help of IAA and NAA alone or with BA (Table 4 and 5).

Impact of IBA + IAA + NAA

When shoot apices of the same selected cultivar obtained through MTSTC and SBSTC were cultured on MS(×½) supplemented with IBA + IAA NAA (2.0-5.0µM), they produced callus at cut ends (CCE). The callus produced by the shoots obtained through MTEC was loose light brown massive callus (LLMC) while that produced by those obtained through SBEC was loose and light yellow (LYLC) (Tables 4 and 5).

Best root initiation and elongation to obtain complete plantlets from the shoots derived from explants was observed on MS (1/2) supplemented with IBA (2.5µM) + PG(10µM) which corroborates with the results of (Zimmerman *et al.,* 1989) has reported use of PG with IBA for root initiation. (Pawalicki.,1992) and (Puentae., 1992) reported strong inhibition of rooting in microshoots by BA. They also reported that splitting of shoot segments enhanced rooting on suitable media. BA has been found to show strong inhibition in rooting of shoots when applied alone. Present work has also found BA to show strong inhibition in rooting. In combination with low auxin (BA 5µM + IBA 2.5µM) normal but delayed rooting was observed. It is probably because of low endogenous cytokinin level in the shoots.

Fig. 01 Establishment of shoot tips on MS (×½)+BA(4.0µM)+PG(10µM) Five weeks after inoculation

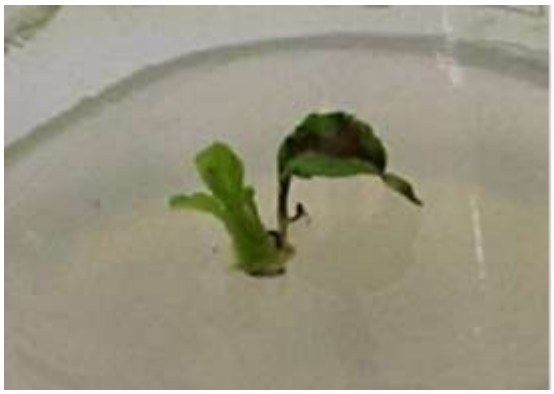

Fig. 02 Shoot multiplication on MS(×½)+BA(4.0µM)+PG(10µM) After eight weeks of culture period

Fig. 03 Further shoot multiplication of subcultured shoots on MS(×½)+BA(4.0µM)+PG(10µM) 14 weeks after culture period

Fig. 04 Subcultured shoots showing further proliferation and multiplication on MS(×½)+BA(4.0µM)+PG(10µM) After eighth subculture

Fig. 05 Subcultured shoot on rooting medium MS (×½)+BA(2.5µM)+PG(10µM) After one week of subculture

Fig. 06 Normal rooting of subcultued shoot on MS (×½)+IBA(2.5µM)+PG(10µM) After three weeks of subculture

PLATE – A

Fig.1 Multiplication of subcultured shoots on MS (×½)+BA(5µM)+PG (10µM) after 16 months of culture period

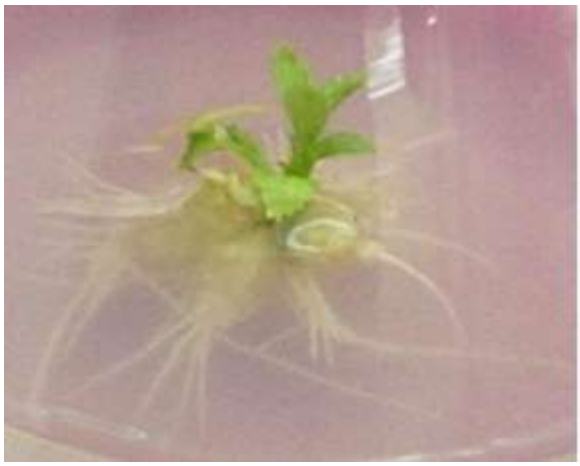

Fig.2 Rooting of sub cultured shoots on MS (×½)+BA(4µM)+PG(10µM). After 16 months of culture period

Fig.3 Micro plant in a thumb pot containing soil-peat Mixture

PLATE – B

Fig.1 Establishment of shoot tips on MS (×½)+BA(4µM)+PG(10µM) Three days after inoculation

Fig. 02 Shoot multiplication on MS (×½)+BA(4µM)+PG(10µM) Four weeks of culture period

Fig. 03 Subculture for further shoot multiplication on MS (×½)+BA(4µM)+PG(10µM) Eight weeks after culture period

Fig. 04 Subculture of individual shoots for rooting on MS (×½)+IBA(2.5µM)+PG (10µM) after sixth subculture

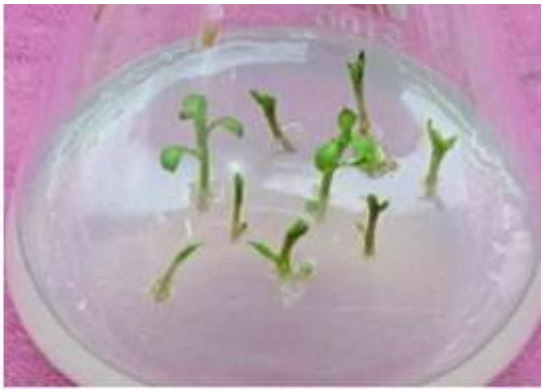

Fig. 05 Normal rooting of subcultured shoots on MS (×½)+IBA(2.5µM)+PG(10µM) After two weeks of subculture

Fig. 06 Complete plantlets thus obtained in thumbpots containing soil-peat (1:1) mixture for hardening.

PLATE – C

Table.3 Impact of cytokinins (BA and TDZ)* on the shoot apices from mature trees of Galmast cultivar of apple cultured in vitro on MS (half-strength) nutrient medium

S.No	Phytohormones (µM)	Nature of Response CH	% of response CH	Shoot Number Mean±SD**/ Nature of callus CH
01	CONTROL	NR	0	NA
02	BA (0.5)	NR	0	NA
03	BA (1.0)	NR	0	NA
04	BA (1.5)	NR	0	NA
05	BA (2.0)	CCE	5	LYC
06	BA (2.5)	CCE	5	LYC
07	BA (3.0)	CCE	10	LYC
08	BA (3.5)	CCE	20	LYC
09	BA (4.0)	ASP	30	12±0.82
10	BA (4.5)	ASP	35	13±0.72
11	BA (5.0)	ASP	40	14±0.72
12	BA (0.5) + PG (10)	NR	0	NA
13	BA (1.0) + PG (10)	NR	0	NA
14	BA (1.5) + PG (10)	NR	0	NA
15	BA (2.0) + PG (10)	NR	0	NA
16	BA (2.5) + PG (10)	CCE	20	LYC
17	BA (3.0) + PG (10)	CCE	25	LYC
18	BA (3.5) + PG (10)	CCE	20	LYC
19	BA (4.0) + PG (10)	ASP	90	42±0.71
20	BA (4.5) + PG (10)	ASP	75	40±0.72
21	BA (5.0) + PG (10)	ASP	70	36±0.82
22	TDZ (0.5)	NR	0	NA
23	TDZ (1.0)	NR	0	NA
24	TDZ (1.5)	NR	0	NA
25	TDZ (2.0)	CCE	15	LCC
26	TDZ (2.5)	CCE	15	LCC
27	TDZ (3.0)	CCE	20	LCC
28	TDZ (3.5)	CCE	15	LCC
29	TDZ (4.0)	CCE	30	LCC
30	TDZ (4.5)	CCE	30	LCC
31	TDZ (5.0)	CCE	25	LCC
32	TDZ (0.5) + PG (10)	NR	0	NA
33	TDZ (1.0) + PG (10)	NR	0	NA
34	TDZ (1.5) + PG (10)	NR	0	NA
35	TDZ (2.0) + PG (10)	NR	0	NA
36	TDZ (2.5) + PG (10)	NR	0	NA
37	TDZ (3.0) + PG (10)	CCE	15	LCC
38	TDZ (3.5) + PG (10)	CCE	15	LCC
39	TDZ (4.0) + PG (10)	CCE	15	LCC
40	TDZ (4.5) + PG (10)	ASP	25	14±2.11
41	TDZ (5.0) + PG (10)	ASP	30	12±0.32

CH - Galmast, CCE - Callus at Cut End; LYC-Loose Yellowish brown Callus; LCC-Loose Creamy Callus; NR - No Response ASP – Adventitious Shoot Proliferation; NA- Not Applicable; * - Kinetin was also initially used but no response was observed; Mean±SD**- Twenty replicates/ treatment. The highlighted values denote significant trials at the level $\alpha \leq 0.05$.

Table.4 In vitro response of subcultured shoots (obtained from mature trees) of Galmast cultivar of apple to auxins for rooting on MS(half strength nutrient medium

S. NO	Phytohormones (µM)	Nature of Response	Percentage of response	Root Number Mean±SD*/Nature of Callus
	Control	CH	CH	CH
01	IBA (0.5)	NR	NR	NR
02	IBA (1.0)	NR	0	NR
03	IBA (1.5)	CCE	05	CYC
04	IBA (2.0)	CCE	05	CYC
05	IBA (2.5)	ARF	20	
06	IBA (3.0)	ARF	60	
07	IBA (3.5)	CR	40	13±0.82
08	IBA (4.0)	CR	40	12±0.71
09	IBA (4.5)	CR	40	11±0.82
10	IBA (5.0)	CR	35	23±0.81
11	IBA (0.5) + PG (10)	CR	25	24±0.72
12	IBA (1.0) + PG (10)	NR	0	NA
13	IBA (1.5) + PG (10)	NR	0	NA
14	IBA (2.0) + PG (10)	NR	0	NA
15	IBA (2.5) + PG (10)	CR	35	13±0.81
16	IBA (3.0) + PG (10)	CRA	100	12±0.68
17	IBA (3.5) + PG (10)	RF	65	15±0.65
18	IBA (4.0) + PG (10)	CR	60	13±0.65
19	IBA (4.5) + PG (10)	CR	60	13±0.65
20	IBA (5.0) + PG (10)	CR	65	12±0.68
21	IAA (0.5)	CR	35	14±0.77
22	IAA (1.0)	CR	0	NA
23	IAA (1.5)	NR	0	NA
24	IAA (2.0)	NR	0	NA
25	IAA (2.5)	NR	0	NA
26	IAA (3.0)	NR	0	NA
27	IAA (3.5)	CEE	10	CYC
28	IAA (4.0)	CEE	10	CYC
29	IAA (4.5)	CEE	30	CYC
30	IAA (5.0)	CEE	30	CYC
31	NAA (0.5)	CEE	25	CYC
32	NAA (1.0)	NR	0	CYC
33	NAA (1.5)	NR	0	NA
34	NAA (2.0)	NR	0	NA
35	NAA (2.5)	NR	0	NA
36	NAA (3.0)	NR	0	NA
37	NAA (3.5)	CEE	0	NA
38	NAA (4.0)	CEE	21	CYC
39	NAA (4.5)	CEE	35	CYC
40	NAA (5.0)	CEE	15	CYC
41	IBA+NAA+IAA (0.5)	CEE	25	CYC
42	IBA+NAA+IAA (1.0)	NR	25	CYC
43	IBA+NAA+IAA (1.5)	NR	0	NA
44	IBA+NAA+IAA (2.0)	CEE	0	NA
45	IBA+NAA+IAA (2.5)	CEE	90	NA
46	IBA+NAA+IAA (3.0)	CEE	100	LLMC
47	IBA+NAA+IAA (3.5)	CEE	55	LLMC
48	IBA+NAA+IAA (4.0)	CEE	65	LLMC
49	IBA+NAA+IAA (4.5)	CEE	60	LLMC
50	IBA+NAA+IAA (5.0)	CEE	55	LLMC
51	BA +IBA/NAA/IAA (0.5)	CEE	50	LLMC
52	BA +IBA/NAA/IAA(1.0)	NR	0	LLMC
53	BA +IBA/NAA/IAA (1.5)	NR	0	NA
54	BA +IBA/NAA/IAA (2.0)	NR	0	MA
55	BA +IBA/NAA/IAA (2.5)	NR	0	NA
56	BA +IBA/NAA/IAA (3.0)	NR	0	NA
57	BA +IBA/NAA/IAA (3.5)	NR	0	NA
59	BA +IBA/NAA/IAA (4.0)	NR	0	NA
60	BA +IBA/NAA/IAA (4.5)	NR	0	NA
61	BA +IBA/NAA/IAA (5.0)	CEE	15	GWMHC

, CH - Galmast, CCE - Callus at Cut End; CYC-Creamy Yellow Callus; LLMC – Loose Light Brown and Massive Callus; ARF–adventitious Root Formation; CR – Callose Roots; NR - No Response; NA- Not Applicable; GWMHC – Greenish White Hard Massive Callus; Mean±SD*: Twenty replicates/treatment. The highlighted values (represented by letters a –n) denote significant results at the level $\alpha \leq 0.05$.

Table.5 Impact of cytokinins (BA and TDZ)* on the shoot apices from mature trees and *in vitro* grown seedlings of Galmast cultivar of apple cultured *in vitro* on MS (half-strength) nutrient medium

S. No	Phytohormones (µM)	Nature of Response	Percentage of response	Shoot Number Mean±SD** / Nature of Callus
1	Control	NR	0	NR
2	IBA (0.5)	NR	0	NA
3	IBA (1.0)	CCE	5	CWLC
4	IBA (1.5)	CCE	10	CWLC
5	IBA (2.0)	ARF	20	08±0.72
6	IBA (2.5)	ARF	85	10±0.68
7	IBA (3.0)	CR	35	10±0.88
8	IBA (3.5)	CR	15	12±0.64
9	IBA (4.0)	CR	15	10±0.70
10	IBA (4.5)	CR	10	14±0.82
11	IBA (5.0)	CR	10	23±0.82
12	IBA (0.5) + PG (10)	NR	0	NA
13	IBA (1.0) + PG (10)	NR	0	NA
14	IBA (1.5) + PG (10)	NR	0	NA
15	IBA (2.0) + PG (10)	CR	35	11±0.65
16	IBA (2.5) + PG (10)	ARF	70	8±0.70
17	IBA (3.0) + PG (10)	CR	70	10±0.78
18	IBA (3.5) + PG (10)	CR	65	15±0.82
19	IBA (4.0) + PG (10)	CR	65	16±0.82
20	IBA (4.5) + PG (10)	CR	50	16±0.71
21	IBA (5.0) + PG (10)	CR	0	16±0.77
22	IAA (0.5)	NR	0	NA
23	IAA (1.0)	NR	0	NA
24	IAA (1.5)	NR	0	NA
25	IAA (2.0)	NR	0	NA
26	IAA (2.5)	NR	0	NA
27	IAA (3.0)	NR	0	NA
28	IAA (3.5)	NR	30	NA
29	IAA (4.0)	CCE	30	CWLC
30	IAA (4.5)	CCE	30	CWLC
31	IAA (5.0)	CCE	0	CWLC
32	NAA (0.5)	NR	0	NA
33	NAA (1.0)	NR	0	NA
34	NAA (1.5)	NR	0	NA
35	NAA (2.0)	NR	0	NA
36	NAA (2.5)	NR	10	NA
37	NAA (3.0)	CCE	10	CWLC
38	NAA (3.5)	CCE	35	CWLC
39	NAA (4.0)	CCE	15	CWLC
40	NAA (4.5)	CCE	25	CWLC
41	NAA (5.0)	CCE	0	CWLC
42	IBA+NAA+IAA (0.5)	NR	0	NA
43	IBA+NAA+IAA (1.0)	NR	0	NA
44	IBA+NAA+IAA (1.5)	NR	75	NA
45	IBA+NAA+IAA (2.0)	CCE	100	LYLC
46	IBA+NAA+IAA (2.5)	CCE	50	LYLC
47	IBA+NAA+IAA (3.0)	CCE	60	LYLC
48	IBA+NAA+IAA (3.5)	CCE	65	LYLC
49	IBA+NAA+IAA (4.0)	CCE	25	LYLC
50	IBA+NAA+IAA (4.5)	CCE	55	LYLC
51	IBA+NAA+IAA (5.0)	CCE	50	LYLC

CH- Gala mast, CCE - Callus at Cut End; CWLC-Creamy White Loose Callus; LYLC – Light Yellow Loose Callus; ARF–adventitious Root Formation, CR – Cellose Roots; NR - No Response, NA- Not Applicable, Mean±SD*: Twenty replicates/treatment

All the soil samples collected from In Delicious apple cultivars rooting percentage and the number of roots in microshoots increased with increase in the number of subcultures (Sriskandrajah *et al.*, 1982). The P studies have also shown similar pattern of rooting response.

Hardening of the microplants

The microplants produced through shoot tip culture were taken out of culture vials very

carefully, roots were washed under running water to remove agar and then they were transferred to pots containing soil mix (soil – peat 1:1). Potted plants were covered by perforated polyethene bags and kept under continuous observation. The plants were misted after regular intervals to maintain maximum humidity (90-100%) under laboratory conditions (Plate-A, Fig. 3; B, Fig. 6; C, Fig. 6). The survival rate was found to be 80% in the plants obtained through MTEC and 95% in those obtained through SBEC.

Establishment of Protocol for micro plant production

From aforementioned observations it can be envisaged that the establishment of the explants from mature trees (MTEs) and seedling born explants (SBEs) of Galmast cultivar occurs best on MS(×½) + BA(4.0-5.0µM) + PG (10µM), shoot multiplication occurs best on same medium augmented with BA(4.0µM) + PG (10µM) and root induction occurs best on the medium containing IBA(2.5µM) + PG(10µM). An outline of the complete protocol of shoot tip culture of Galmast cultivar of apple obtained from mature trees has been summarised in Plate-A for its clonal propagation.

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