Indian J. Plant Physiol., Vol. 9, No. 2, (N.S.) pp. 136-144 (April-June, 2004)

FACTORS INFLUENCING *IN VITRO* ESTABLISHMENT OF MANGO SHOOT BUDS

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Received on 7 May, 2003, Revised on 3 June, 2004

SUMMARY

Mango shoot bud explants taken directly from the field grown mature tree face major problems of phenolic exudation and deep seated contamination in establishment of aseptic cultures. Due to these problems, the explants do not survive long enough to respond to culture conditions. The problems of phenolic exudation and microbial contamination were overcome using various sequential pretreatment and different sterilizing agents. The shoots were defoliated in the field and sprayed with disinfectant solution (0.1% bavistin + 0.1% gentamycin) followed by spray of 2 mg/l BA + 2 mg/l GA_3 every alternate day upto 15 days. The shoots were collected in antioxidant solution (ascorbic acid 70 mg/l + citric acid 50 mg/l) and kept for 30 min. followed by running tap water treatment for two hours and stirring in PVP (0.5%) for 1 hour. This was followed by sequential sterilization with 2-3 drops of Tween-20 in distilled water for one hour followed by rinsing with 70% alcohol for 30 seconds and 0.1% $HgCl_2$ treatment for 12-15 min. before inoculating on medium. Subculturing into fresh medium was done on every alternate days up to one week and then weekly. Using these methods 80% sterile cultures were obtained which remained green for 8-10 weeks on MS basal medium supplemented with 3 mg/l indole acetic acid + 1 mg/l kinetin + 200 mg/l adenine sulphate + 1 mg/l thiamine + 1 mg/l riboflavin + 100 mg/l casein hydrolysate + 400 mg/l glutamine + 100 mg/l PVP + 100 mg/l ascorbic acid + 60 mg/l sucrose. Shoot buds of cv. Amrapalli showed the best response with 48.92% cultures remaining green with opening of leaf primordial even after 4 weeks to inoculation. Differentiation of shoot buds into plantlets was achieved but plantlets did not grow beyond 3-4 leaf primordial stage.

Key Words **: Aseptic culture, *in vitro* culture, mango, shoot bud explant.**

INTRODUCTION

India is the major producer of monoembryonic mango, many of which are esteemed for high quality fruits and considered far superior to their polyembryonic counterparts. There is a great demand for planting material of superior mango cultivars. The monoembryonic cultivars do not breed true to type, if propagated from seeds. Thus they are commonly propagated through grafting, which is too slow to meet the increasing demand of genuine superior planting material. With the use of tissue culture techniques, it is possible not only to accelerate the mutiplication of true to type planting material but also to facilitate easy storage and safe exchange of germplasm. Somatic embryogenesis has been reported in several mango cultivars using nuclear tissue as an expalnt and some success has been achieved but the survival of the *in vitro* raised plantlets is very poor (Litz *et al.* 1982, Litz 1988, Jana *et al.* 1994, Ara *et al.* 2000). Moreover the *in vitro* raised plantlets also showed abnormalities as well

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as genetic variation from the mother plant. Therefore, the efforts have been made to standardize protocol for clonal propagation of mango by vegetative explants of mature tree like shoot apex/axillary buds. In this direction the initial reports have been published using two-year-old glasshouse grown plants (Yang and Ludders 1993) but limited shoot proliferation was achieved.

Somatic embryogenesis has also been reported (Litz *et al.* 1985, Jana *et al.* 1994, Ara *et al.* 2000) yet the survival percentage of *in vitro* plantlet is very poor. Under these conditions regeneration using vegetative plant parts like shoot bud becomes obligately. Work was done using 8-16 years old tress (Thomas and Ravindra 1997) but the results were not very encouraging.

Studies using mango shoot buds indicated various problems such as phenolic exudation, medium discoloration, explant browning and endophytically deep-seated contamination, which create hurdles in establishment of cultures. The secondary metabolites, which are strongly oxidized after wounding, cause subsequent catastrophic browning and necrosis leading to death of the explant. The explant fail to survive long enough to allow growth regulators to influence growth response due to microbial contamination. These factors hinder the establishment of aseptic cultures of explant taken from field grown mature tress of mango (Thomas and Ravindra 1997, Padaria *et al.* 1999, Chandra *et al.* 2000). The present study was undertaken to analyse the factors influencing the *in vitro* establishment and response of shoot bud explant and to overcome the problems in successful shoot bud culture.

MATERIALS AND METHODS

Shoot buds were taken from 20 year old mature trees of cultivars Amrapalli, Dashehari, Langra and Chausa from the CISH farm, Rehmankhera, Lucknow and brought to the laboratory in polythene bags. The explants were collected throughout the year so as to observe the influence of season in establishment/response of cultures (Fig. 1). The influence of age and size of the explant on *in vitro* response was studied. The explants of varying size of 0.5 cm to 10 cm (*i.e.* 0.5 cm-1 cm, 1-3 cm, 3-5 cm, 5-7 cm, 7-10 cm) (Fig. 2) and explant of different age groups (Fig. 3) were taken to study its influence on explant response. Fifty explants were taken in each treatment.

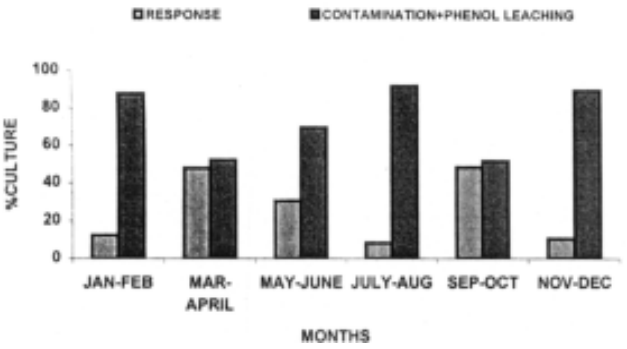

Fig. 1. Effect of season on shoot bud culture of mango (after 4 week)

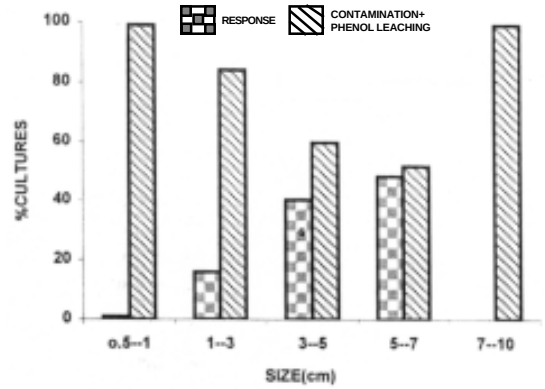

Fig. 2. Effect of explant size on shoot bud culture of mango (after 4 week)

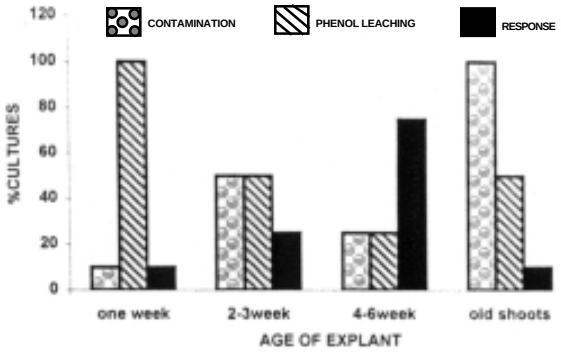

Fig. 3. Effect of different age of explant on shoot bud culture of mango (cvs Dashehari and Amrapalli)

Excessive exudation of phenolic compounds and deep-seated microbial contamination is a serious bottleneck in establishment of aseptic cultures. Different procedures and antioxidants as well as adsorbents were tried to overcome the problem of phenolic exudation (Table 1). Use of sterilizing agents, viz. mercuric chloride ($HgCl_2$), sodium hypochloride (NaoCl), calcium hypochloride ($CaOCl_2$), antibiotics and fungicides as ampicillin, bavistin,

Table 1. Response of antioxidants to control phenol leaching.

Treatment	% of cultures showing no phenol leaching (days/weeks after inoculation)										
	3rd day	5th day	1	2	3	4	5	6	7	8	10
Washing under running tap water two hrs+stirring in 0.5 % PVP for one hr	1.20	2.60	4.20	10.0	12.4	13.0	23.0	26.4	31.0	-	-
Washing under running tap water two hrs+2.5 gm/l PVP to media	2.00	3.60	5.20	10.4	13.6	15.4	24.4	27.4	33.3	34.4	-
Washing under running tap water two hrs+stirring in 0.5% PVP 1 hr + 2.5 gm/l PVP+ 100 mg/l ascorbic acid to media	2.60	4.00	5.80	11.8	14.8	16.4	25.0	28.8	34.4	47.0	51.0
Washing under running tap water two hrs+ stirring 0.5% PVP 1hr + 2.5 gm/l PVP+ 100 mg/l ascorbic acid to media + regular sub-culturing	3.40	5.60	7.00	14.4	17.8	23.8	27.6	38.4	45.5	75.6	80.4
Collecting explants in solution of 70 mg/l Ascorbic acid + 50 mg/l citric acid + washing under Running tap water two hrs+ stirring in 0.5% PVP1 hr,+100mg/l ascorbic acid + 2.5 gm/l PVP to media + regular subculturing	5.6	9.0	19.6	24.4	42.0	57.0	62.0	64.0	76.0	85.0	93.2

Table 2. Effect of surface sterilants and their treatment time on survival of shoot tip explants.

Treatment	Aseptic culture (%) weeks after inoculation					Response/Proliferated explants (%) after 16 weeks
	2	4	8	12	16	
Control (no sterilant)	1	-	-	-	-	contaminated
0.1% $HgCl_2$ (0.1%): 3-5 min.	20	-	-	-	-	contaminated
0.1% $HgCl_2$ 6-8 min	50	45	-	-	-	Tissue remain green initially then became contaminated
0.1% $HgCl_2$ 10-12 min	90	75	-	-	-	Explant turned brown/black within 2 weeks of inoculation
0.1% $HgCl_2$ 15 min	68	65	-	-	-	-do-
0.1% $HgCl_2$ 20 min	70	65	-	-	-	Explants gradually turn brown/black
20% NaOCl 5 min	20	-	-	-	-	NR
20% NaOCl 10 min	30	25	-	-	-	Explants turn dull green
20% NaOCl 20 min	35	30	-	-	-	-do-
10% $CaOCl_2$ 5 min	20	-	-	-	-	Explant turn brown/black
10% $CaOCl_2$ 10 min	40	35	-	-	-	-do-
10% $CaOCl_2$ 15 min	40	35	-	-	-	-do-
0.1% $HgCl_2$ 2 min + 20% NaOCl 10 min	25	18	-	-	-	Expalnt initially green and then contaminated
0.1% $HgCl_2$ 5min + 20% NaOCl 15 min	28	20	-	-	-	-do-

Treatment	Aseptic culture (%) weeks after inoculation					Response/Proliferated explants (%) after 16 weeks
	2	4	8	12	16	
0.1% $HgCl_2$ 6-8 min + 20% NaOCl 20 min	30	25	-	-	-	Expalnt initially green and then contaminated
0.05% Amp + 0.01% Bavistin stirring 2hrs + 0.1 % $HgCl_2$ 4min	45	30	-	-	-	Microbial contamination was reduced but explants turn dull yellow & gradually became brown/black
0.01% Amp + 0.1% Bavastin stirring 2hrs + 0.1% $HgCl_2$ 6-8 min	59	42	30	-	-	-do-
0.05 Amp +0.1% Genta + 0.1% Bavastin stirring 2 hrs + 0.1% $HgCl_2$ 6-8 min	78	72	55	42	18	12% cultures showing proliferation of young leaf primordia contamination reduced explant turn brown/ black gradually after 8 week of culture.
0.1% $HgCl_2$ 0-10 min +0.05 gm/l Bavitin in the medium	72	70	60	-	-	No Response Expalnt turn dull green to pale yellow.
0.05% Amp + 0.1% Genta stirring 2 hrs 0.1% $HgCl_2$ 10-12 min + 0.05 gm/l Bavitin in the medium	80	75	70	-	-	Explants responsive remain green for 6-8 week.
Shoot bud defoliated and sprayed with a disinfected solution 15 days + 0.1% $HgCl_2$ 10-12 min.	91	85	78	72	70	Explants responsive remain green for 8-10 weeks of culture, opening of new leaf in (6%) culture after 12 weeks explants turn brown/black and gradually became dead but no phenol leaching and contaminations was observed even after 16 weeks of culture.

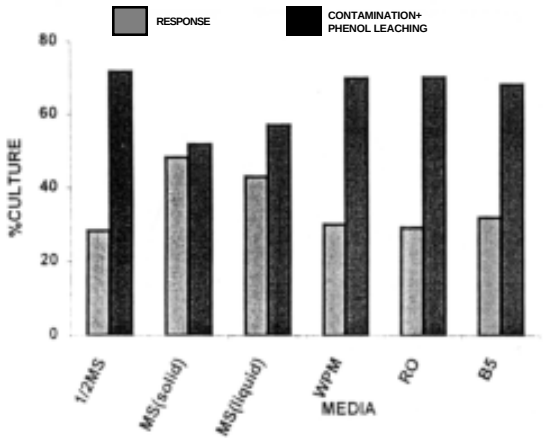

Fig. 4. Effect of basal medium on shoot bud culture of mango (after 4 week)

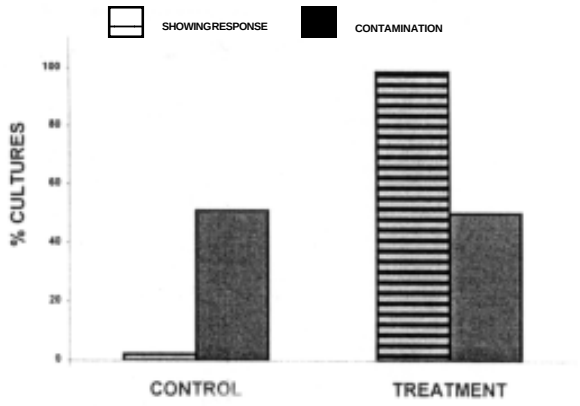

Fig. 5. Effect of explant pretreatment on *in vitro* of mango shoot bud

etc. were tried to overcome the problem of microbial contamination (Table 2). Pre-curing/pre-treatment of the explants when on tree itself was also attempted. This was done by defoliating the growing shoots (Fig. 7) and these were then regularly sprayed with 0.1% bavistin + 0.1% gentamycin (genta) + 0.5% ampicillin (amp) + 0.5% streptomycin (strepto) and on alternate day with growth regulators benzyl N6 adenine (BA-2 mg/l) + gibberellic acid (GA_3-2 mg/l) for 15 days. The explant, after undergoing various steps of pre-treatment was surface sterilized and inoculated singly in culture tubes (15 cm x 2.5 cm) containing 10 ml semisolid media or 5 ml liquid media containing basal media salts supplemented with growth regulators and gelled with 0.8% agar.

Different basal media as B5 (Gamborg *et al.* 1968) Rugini olive medium (RO) (Rugini 1984), Woody plant media (WPM) and MS medium (Murashige and Skoog 1962) were tried to establish the explant (Fig. 4). The sucrose concentration in the media was kept at 6%. MS

media was tried at full concentration and half concentration. Different growth regulators i.e. cytokinis viz., BA, kinetin (kn) and zeatin (zea); auxins viz indole 3-butryic acid (IBA), naphthalene acetic acid (NAA) and indole acetic acid (IAA) and paclobutrazol (paclo) were used to supplement the basal medium for getting desired response (Table 3). Vitamins such as thiamine HCl (B1) and riboflavin (B2) were also added to the medium. Casein hydrolysate (CH) and glutamine (gln) were added as supplementary nitrogen source. The pH of the medium was adjusted at 5.8 and autoclaved at 1.1 kg/cm pressure and 121°C for 15 min. The cultures were incubated at 25 ± 2°C under 16 hours fluorescent light supplemented with incandescent bulbs. The cultures were observed daily and data were recorded. The growth response of cultures such as swelling of axillary buds and initiation of callus

Table 3. Response of different growth regulators on shoot bud culture

Media	Observation	Response
MS+0.50 mg/l Paclo	Tissue remain green but no further growth	+
MS+10.0 mg/l Paclo	Explant responsive, growing of the tip	+
MS+200 mg Paclo	-do-	+
MS+300 mg Paclo	-do-	+
MS+100 mg/l Paclo +10.0 mg/l GA_3+3.0 mg/l BA +3.0 mg/l Charcoal 1	Shoot tip growing, opening of 1-2 leaf primordial	++
MS+ –100 mg/l Paclo +10.0 mg/l GA_3+0.2 mg/l BA +200 mg/l ADS	Explants responsive growing of the tip upto 4-5 weeks, no further growth	+
MS+ –200 mg/l Paclo +200 mg/l ADS+2.0 mg/l BA +0.2 mg/l NAA	-do-	+
½ B_5 major+MS minor+100mg/l Paclo+5.0 mg/l IBA+10 mg/l GA_3	Tissue remains green, slight swelling of axillary buds	+
½ B_5 major+MS minor+100mg/l Paclo+-100mg/l PVP+2mg/l NAA	-do-	+
MS+100mg/l Paclo+400mg/l gln+100mg/l CH+1.0 mg/l IAA+3.0mg/l Kn3.0mg/l 2, 4D	-do-	+
MS+400mg/l gln+100mg/l CH+15mg/l Kn	Tissue remains grew opening of leaf primordial and growing of leaf	+++
MS+400 mg/l gln+100mg/l CH+2.0 mg/l Kn	-do-	++
MS+400 mg/l gln+100 mg/l CH+15 mg/l Kn +5mg/l Zea	Tissue green and responsive swelling at the base, growing of young leaves, 2-3 cm. in size	+++
MS+400 mg/l gln+100 mg/l CH+20 mg/l Kn +5 mg/l Zea	-do-	+++
WPM+1-5mg/l IAA +1-5 mg/l Kn +400mg/l gln+100 mg/l CH	Tissue remains green for 6-8 weeks of culture swelling at the base of the shoots	+
WPM+5-10 mg/l Kn+1-5.0 mg/l Zea +400 mg/l gln +100 mg/l CH	-do-	+
RO+1-5 mg/l AA+1-5 mg/l Kn+400 mg/l gln +100 mg/l CH	Shoot tips remain green and responsive but no further growth	+
RO+400 mg/l BA+1-5 mg/l Kn +400mg/l gln+100 mg/l CH+100 mg/l PVP	-do-	+
RO+1-5mg/l BA+1-5 mg/l BA+400 mg/l gln+400 mg/l CH+100 mg/l PVP+100 mg/l Ascorbic Acid	-do-	+
MS+1.0 mg/l IAA+3.0 mg/l Kn+1.0 mg/l Vit B1 & B2+200 mg/l ADS+400 mg/l gln+400 mg/l CH+100 mg/l PVP +100 mg/l Ascorbic acid + 60 gm/l sucrose	Explants remain green upto 8 to 10 weeks of culture, opening of 3 to 4 new leaf primordial swelling of axillary buds	+++

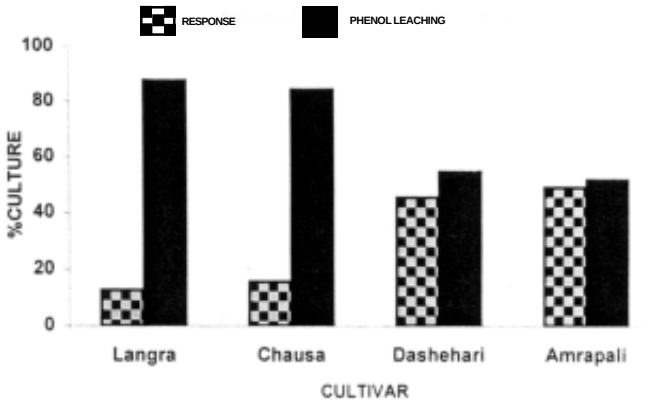

Fig. 6. Effect of genotype on shoot bud culture of mango (after 4 week)

Fig. 7. Defoliation of mango shoots in field

and emergence of leaf primordial was observed, as cultures remained green.

RESULTS AND DISCUSSION

Establishment of aseptic cultures of mango shoot bud explant from mature adult trees was difficult due to deep seated endophytic microbial contamination. Different sterilizing agents, viz., antibiotics, fungicides like mercuric chloride, sodium hypochloride, calcium hypochloride, ampicillin, bavistin, etc. were used to overcome this problem. The various sterilizing agents tried in varying concentration only delayed the expression or reduced the

Fig. 8. *In vitro* growth response of mango shoot bud

intensity of the contaminants as contamination was observed even in 8-10 weeks old cultures. 80% aseptic cultures were obtained when the explants were treated with 0.1% $HgCl_2$ for 10-12 min but these cultures survived for 4 weeks only. Within 2 weeks of inoculation the explant turned black and gradually died. Addition of antibiotic and fungicide was effective in controlling microbial growth, but the cultures did not show any response and could not survive beyond 8 weeks. Shaking the explants in antibiotic and fungicide suspension followed by 0.1% $HgCl_2$ treatment for 10 min, made 12% explants responsive but after 8 weeks all the cultures turned black and died. It was found that pretreating the explant on the tree itself wherein, the shoot buds were defoliated and regularly sprayed with disinfectant solution (0.1% bavistin + 0.1% gentamycin + 0.5% ampicillin + 0.5% streptomycin) and on alternate day with growth regulators

solution (2 mg/l BA + 2 mg/l GA3) for 15 days, greatly improved the percent aseptic cultures. Fifty percent cultures were totally free from phenol exudation and contamination. 50.92% cultures were responsive after 4 weeks of inoculation. In control only 2.20% cultures were responsive whereas 98.8% cultures were contaminated or there was phenol exudation (Fig. 5). Pretreated/pre-cured explants when surface sterilize with 0.1% $HgCl_2$ for 10-12 min resulted in 91% sterile cultures. The explants were responsive and remained green for 8-10 weeks, with the opening of new leaf primordia in 6% cultures. After 12 weeks explants turned brown/black and gradually died. Even after 16 weeks, though the explants died yet 70% cultures were free of contamination (Table 2). Similar mercuric chloric treatment on non-precured buds was not as effective.

In most of the perennial woody plants on wounding phenolics are released which can be oxidised to black quinones and polymerized material which cause discoloration of medium and death of plant. Debergh Read (1991), George and Sherrington (1984) have listed several ways to prevent medium and explant blackening which have been found to be useful in different woody perennials as in cashew (Mneney and Mantell 2001). In case of mango, Thomas and Ravindra (1997) found that use of charcoal on the medium was beneficial for explant survival but other adsorbents and antioxidants did not offer any advantages. In our experiment, phenolic compounds leaching was controlled in cultures upto 10 weeks with sequential use of different antioxidants on precured shoot bud explant. It was found that collecting the explant in an antioxidant solution of ascorbic acid (70 mg/l) + citric acid (50 mg/l) + washing under running tap water for two hours + shaking in polyvinyl pyrollidone (PVP 0.5%) for 1 hour and addition of ascorbic acid (100 mg/l) and PVP (2.5 mg/l) into the medium + regular sub culturing initially on alternate days upto one week and finally weekly was effective. It was observed that when the explant was made to undergo the above protocol systematically, it resulted in complete control of phenolic compound leaching (Table 1). Even after 10 weeks there was no phenolic exudation though the cultures were non-responsive and gradually died. By 16^{th} week after inoculation, 100% cultures had died. Besides microbial contamination and phenolic exudation, a number of other factors influenced establishment of axenic mango shoot bud cultures.

Genotype

Shoot buds of cultivars Amrapalli showed the best response as 48.92% cultures remained green with opening of leaf primordia even after 4 weeks of inoculation followed by Dashehari (45.33%), Chausa (15.62%) and Langra (12.28%). Maximum phenolic exudation and contamination (87.72%) was observed in shoot buds of variety Langra followed by 84.38% in Chausa, 54.67% in Dashehari and 51.80% in Amrapalli (Fig. 6).

Age of shoot bud

4-6 weeks shoots with green foliage and dark green stem showed best response with least phenol leaching and contamination. These shoots were found ideal for establishment of culture as at this stage/age they are physiologically very active. 1-2 week old leaves i.e. just emerging shoot with reddish brown tender leaves and stem showed maximum explant browning and phenolic exudation where as maximum contamination was found in old shoots with dark green mature foliage and thick brown stem. Explants taken from young seedlings showed least phenolic exudation and contamination with poor growth response (Fig. 3).

Seasonal effect

Seasonal effect was observed when the buds were collected in the month of March-April and Sep.-Oct. The cultures were responsive and microbial contamination was less. Explants collected during mid June to Aug. showed maximum contamination while the explant collected during other period showed more phenol leaching, explants turned brown sooner and showed poor growth response (Fig. 1). Similar seasonal variation in *in vitro* response to explants has also been observed in other plants as papaya (Litz and Conover 1991), guava (Amin & Jaiswal 1998). In case of mango, Thomas and Ravindra (1997) reported June-Aug. as ideal period for *in vitro* response for mango shoot buds in Bangalore, India. The difference in geographical location of the two places may have resulted in difference in the season for best response for *in vitro* mango shoot bud culture.

Size of the explant

The size of the explant is very important for establishment of the cultures as it is related to endogenous microbial load and presence of secondary metabolites that cause catastrophic browning and necrosis of explant in cultures. The explant 5-7 cm length, responded best in *in vitro* in our studies. Similar results have been reported by Thomas and Ravindra (1997) in mango. Smaller explants were relatively free of contaminants and problem of browning was also only marginal, but successful establishment of cultures was poor. Shoot buds with tender leaves were free of contamination but the problem of necrotic browning due to wounding was extensive. Mango shoot bud explants of size 5-7 cm were best for establishment of cultures as 51.42% cultures were devoid of contamination, phenol leaching problem and 48% cultures were responsive as they remained green with the opening of 2-3 leaves. Explants of size of 7-10 cm as well as 0.5-1 cm sizes were not able to establish *in vitro* due to contamination and phenol leaching (99% cultures). Cultures of explant 3-5 cm size were successful in *in vitro* establishment but the cultures did not grow and just remained green (Fig. 2).

Basal Medium

Media did not significantly affect the establishment of mango shoot and culture, though among all the media tried, full MS (semisolid) responded the best. Cultures were responsive (48%) and devoid of contamination and phenolic exudation (52%) in this medium. Among all the basal media tried, ½ MS and RO medium were not found to be suitable for mango shoot bud culture (Fig. 4).

Growth regulators

Basal media were supplemented with different growth regulators to get shoot bud differentiation response in mango (Table 3). MS basal medium supplemented with 3 mg/l indole acetic acid + 1 mg/l kinetin + 200 mg/l adenine sulphate + 1 mg/l thiamine + 1 mg/l riboflavin + 100 mg/l casein hydrolysate and 400 mg/l glutamine was observed to give the best response of shoot bud differentiation. Explants remained green upto 8-10 weeks and there was opening of 2-3 leaf primordia and swelling of axillary bud. Paclobutrazol at different concentrations (0.5 mg/l, 10 mg/l, 200 mg/l and 300 mg/l) was used in the medium and it did give positive response but the cultures did not grow beyond opening of 1-2 leaf primodia (Fig. 8).

This study reveals that pretreating shoot bud of defoliated adult trees with disinfectant solution (0.1% bavistin + 0.1% gentamycin + 0.5% ampillicin + 0.5% streptomycin) and on alternate day with growth regulator solution of 2 mg/l BA + 2 mg/l GA_3 for 15 days controlled the contamination problems. Collecting the explants in an antioxidants solution of ascorbic acid 70 mg/l + citric acid 50 mg/l, washing them under running tap water for 1 hr. and addition of ascorbic acid 100 mg/l and PVP 2.5 mg/l into the medium helped in controlling phenolic leaching problem. Thus this study identifies a protocol for establishment of mango shoot bud cultures from field grown mango trees.

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