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Effect of different factors on *in vitro* shoot tip culture establishment in mango

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ABSTRACT

The individual effects of pre-treatment, explant size, explant age, explanting season, basal salt medium formulation and growth regulators were investigated on shoot *in vitro* culture establishment in monoembryonic mango cvs. Amrapali and Pusa Arunima. Endophytic microbial contamination and rapid browning of explant and culture medium were the major factors, which delimited the explant establishment. Regardless of genotype and duration, pre-treatment comprising etiolation + spray of imidazole + agitation in 0.2% PVP solution before culture was found best with regards to improved culture establishment and reduced infection of shoot cultures. The smaller explants exhibited higher medium browning compared to longer ones. The highest explant survival and least browning were recoded when 4.0 cm long explant were employed. Likewise, explants excised from older shoots, i.e. from 3 to 4-month-old current season shoots registered higher survival in comparison to one week or 3-4 week-old explants. Explants inoculated on medium comprising B5 macro-salts excluding $(NH_4)_2SO_4$ and $(NH_4)_2NO_3$ plus MS micro-salts and organics supplemented with 0.5 mg l^{-1} NAA plus 2.0 mg l^{-1} BAP in March were found superior over other combinations.

Key words: Mango, pre-treatment, *in vitro* culture, survival, microbial contamination.

INTRODUCTION

Most of the cultivated mango varieties are the chance seedlings and are conventionally propagated by vegetative means such as air-layering or grafting on to indiscrete seeding rootstocks. This practice results in subtle changes in horticultural characteristics of any scion variety owing to stionic effects. Though, this approach is slow and labour intensive, it has shown a very high degree of success. However, for the rapid multiplication of any newly released variety for which demand for quality plant material is very high or for an 'elite genotype' selected by a breeder, this method can not be perceived efficient. It would be ideal to collect explants from healthy and well characterized donor plants. However, the choice of donor plants may be limited particularly in mango, where successful regeneration is generally observed in immature zygotic nucellar embryos/ (juvenile tissue), while other explants such as shoot tip, nodal segment, leaf (mature tissues), etc. showed limited success. The major reasons being the juvenility of embryo and nucellar tissues. Though, the success is greatly influenced by genotype; nevertheless, growth status of donor plants; and developmental stage of the embryo on isolation (Laxmi *et al*., 14) are also of immense importance. Therefore, sometimes it becomes inevitable to use mature tissues as explant in former case is dependent on a narrow window of opportunity during the year in which immature fruits are at the appropriate stage for explanting the nucellus. Earlier, Thomas and Ravindra (19) attempted to establish shoot tip culture in some mango genotypes. Their study indicated that several problems such as phenolic exudation, medium discolouration and explant browning are interrelated and are influenced by different factors like medium, genotype, explant, season and decontamination treatment. Browning was overcome by use of different media additives. However, the deep seated and systemic microbial contamination could not be checked completely. Furthermore, frequent decontamination treatments to explant often stimulated the phenolic exudation in the medium. In this study, we explored the potentiality of a triazole, which hitherto has been used as a growth regulator, as fungistatic. The present study was undertaken to investigate the effects of explant size, explanting season, medium, growth regulators and cultivar on shoot cultures mango cultivars so as to optimize culture conditions for *in vitro* propagation of mango.

MATERIALS AND METHODS

In order to asses the potentiality of *in vitro* culture establishment of nodal segments collected from field-grown mango trees, the explants were severed and agitated in a solution containing 2.0 g l^{-1} bavistin and 200 mg l^{-1} 8-hydroxy quinnoline citrate (8-HQC) for

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2 h. The explants were collected from newly emerged flush (1-week-old) from two monoembryonic genotypes Amrapali and Pusa Arunima during September unless otherwise specified. Shoot segment culture establishment was tried in three months, i.e., January, March and September. For standardization of season of explanting current season (3-4 month-old) shoots were used as explant. The etiolation was done by covering the new shoots using perforated black polythene bags (200 gauge) for a week before explant collection. The explants were cultured onto B_5 medium supplemented with 1.0 mg l^{-1} NAA plus 1.0 mg l^{-1} BAP unless otherwise specified. To screen out the best medium, five different types of media formulations *viz.,* full-strength Murashige and Skoog's (16), half-strength MS, full-strength B5 (Gamborg *et al.*, 11), half-strength B5 and B5 macro-salts excluding $(NH_4)_2SO_4$ and $(NH_4)_2NO_3$ plus MS micro-salts and organics abbreviated as MS, 1/2 MS, B5, 1/2 B5 and B5(-), respectively were selected for the studies. Likewise, growth regulator combination 0.5 mg l^{-1} NAA plus 2.0 mg l^{-1}BAP, 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA + 10.0 mg l^{-1} kinetin abbreviated as G1, G2 and G3, respectively were also tried for culture establishment. The pH of media was adjusted to 5.8 ± 0.5 using 0.1 N HCl or 0.1 N KOH. The medium was heated after the addition of agar-agar (7.0 g l^{-1}) in order to homogenize and then dispensed into test tubes (15 ml).

The stock plants were given pre-treatments (presented in Table 1) and assessed for their biochemical status. *In vivo* levels of total phenols (Sadasivam and Manickam, 17) and polyphenol oxidase (Valero *et al.*, 20) were estimated at pre-culture stage. Later, the explants were washed under running tap water for 15-20 min. followed by washing in Teepol® solution and finally rinsing with double-distilled water. Nodal segments were prepared by defoliating and retaining the petiole. The ends of explants were cut in such a manner that upper cut was given just above the node, while the lower one at about one cm below it. Explants of different sizes 1.0, 2.0, 3.0 and 4.0 cm were employed for culture. After proper washing and pre-treatments, the explants were transferred in a laminar flow onto a sterile medium containing conical flask (250 ml). Two rinsing with autoclaved distilled water were given. Surface sterilization was carried out by agitating explants in 70% alcohol (v/v) for 30 sec. followed by 0.1% $HgCl_2$ solution for 4 min. Three to four rinses with pre-autoclaved double-distilled water was given in order to remove residual toxicity of disinfecting agent. Thereafter, the explants were air-dried onto sterile tissue paper cushion in a petridish. The surface sterilized explants were inoculated onto medium as per treatments.

The experiments were laid out in a factorial completely randomized block design with three replications and 50 explants comprised a unit for each treatment. The percentage data were subjected to arc sin $\sqrt{}$% transformation before carrying out the ANOVA.

RESULTS AND DISCUSSION

It is revealed from the present studies that mean value of per cent culture establishment was markedly higher, while microbial contamination was lower in pre-treated explants than non-etiolated control. Irrespective of genotype and duration, maximum explant survival and minimum contamination were noticed in etiolated trees sprayed regularly with imidazole under field conditions and given PVP agitation before inoculation (87.54%), while the minimum survival was noticed in control, i.e. T_0 (67.50%) (Fig. 1). Likewise, in terms of microbial contamination too, T_5 was found superior over other treatments (Fig. 3).

A significant increase in aforesaid parameters was observed when etiolation coupled with the application of imidazole was made on stock plants. This could be attributed to multifarious role of imidazole, which is classified as a triazole compound. Triazoles are known for exerting a gamut of influence on plants. The primary action include (i) inhibition of GA biosynthesis and thereby, acting as stress protectants (Guoping, 12); (ii) interference with biosynthesis of ergosterols (an indispensable component of fungal membranes), and

Table 1. Pre-treatments for enhancement of *in vitro* culture establishment of shoot cultures.

Pre-treatment	Method
T_0	Non-etiolated control.
T_1	Etiolation of stock plants with perforated black polythene bags (200 gauge) for 7 days and four sprays of 2.0 g l^{-1} bavistin on alternate days.
T_2	Four sprays of imidazole (2.0 g l^{-1}) on alternate days.
T_3	Etiolation + spray of imidazole (T_2).
T_4	Etiolation + spray of imidazole + antioxidants agitation (ascorbic acid @ 100 mg l^{-1} + citric acid @ 50 mg l^{-1}).
T_5	Etiolation + spray of imidazole + agitation with 0.2% PVP.

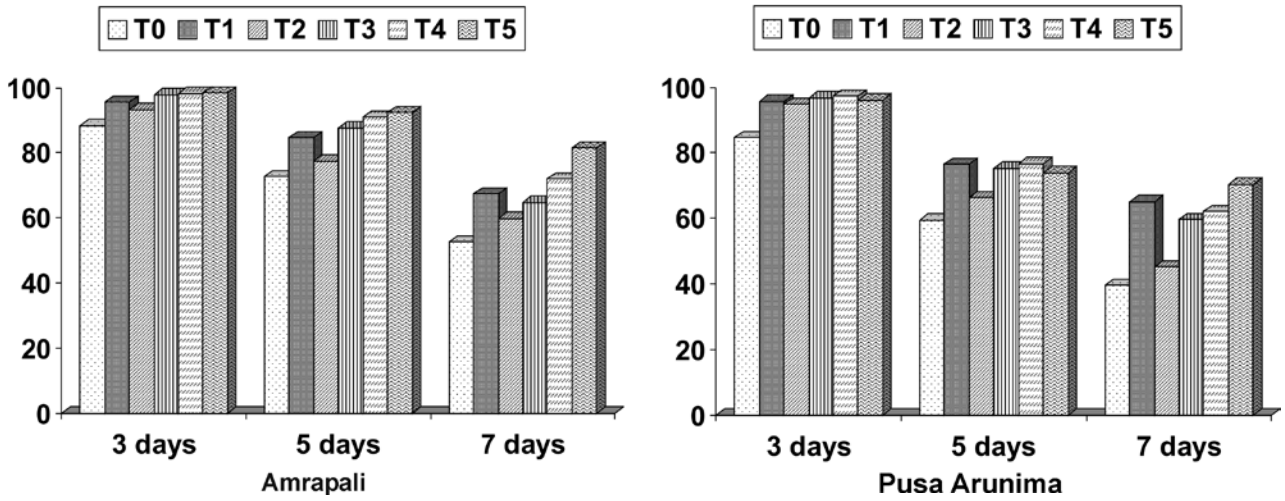

Fig. 1. Effect of different pre-treatments on culture establishment (%) of shoot tip explants in mango.

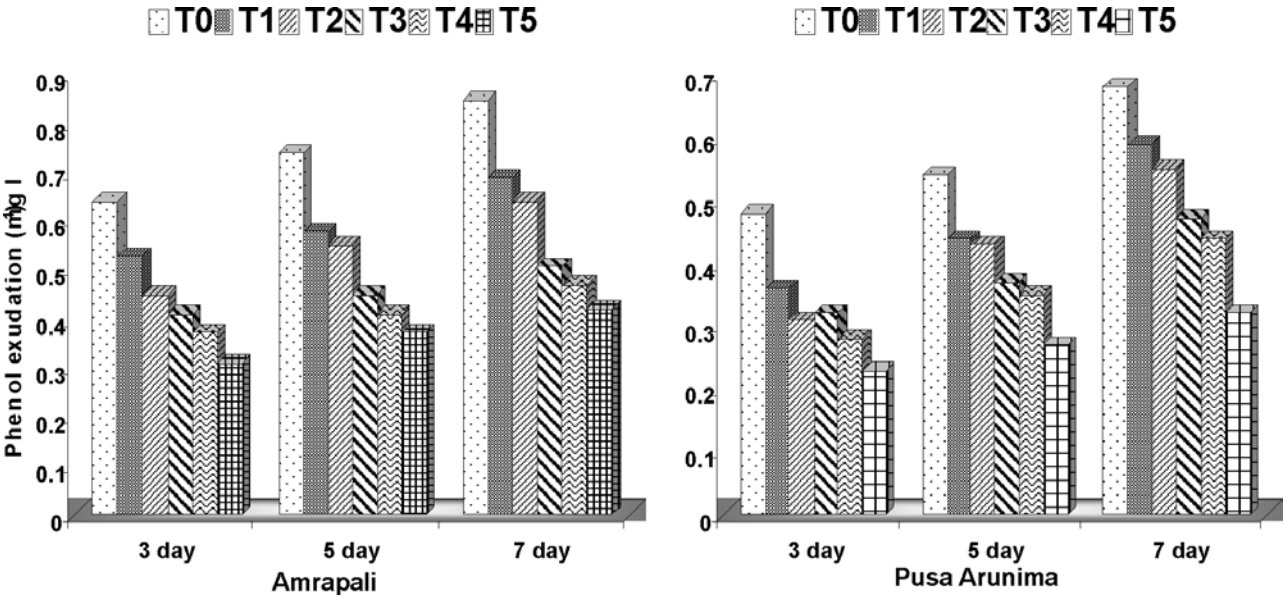

Fig. 2. Effect of different pre-treatments on *in vitro* phenol exudation of mango shoot explants.

inhibition of this sterol leads to loss of membrane integrity and ultimately death of fungal cells (Koller, 13).

The fungal contaminants were pre-dominant *in vitro* culture of mango shoots. They appeared at early stages of culture initiation, while bacterial contaminants appeared at later stages of culture growth. In most of the contaminated cultures, infection first appeared at the apex of shoot explants. This is owed to restriction of sterilant penetration by arrangement of apical leaves and close-set scales in mango (Thomas and Ravindra, 19). Therefore, surface sterilization alone did not give satisfactory results. Being systemic in nature, imidazole sprays helped in eliminating fungal contaminants protected by whorl of leaves and scales. Field spray of mango shoot buds with systemic sterilants such as bavistin and gentamycin prior to culture initiation was earlier recommended by Chandra *et al.* (4).

The enhanced survival in etiolated explants can safely be assigned to reduction of subsequent phenol exudation *in vitro*; though, *in vivo* phenol and polyphenol oxidase levels increased in stock plants sprayed with imidazole (Table 2) as triazoles are found to enhance phenol level in plant tissues (Fletcher *et al.*, 6). The higher survival due to imidazole treatment could be

Table 2. Biochemical status of mango shoot explants in response to different pre-treatments at pre-culture stage.

Treatment	Total phenols (mg g^{-1} F.W.)			Catecholase activity (Δ400 mg^{-1} protein min^{-1})			Cresolase activity (ΔA 400 mg^{-1} protein min^{-1})		
	Genotype			Genotype			Genotype		
	Amrapali	Pusa Arunima	Mean	Amrapali	Pusa Arunima	Mean	Amrapali	Pusa Arunima	Mean
T_0	20.83	22.26	21.55	2398.00	2516.00	2457.00	1823.32	2018.60	1920.96
T_1	18.23	19.73	18.98	1808.10	2100.37	1954.23	1209.06	1480.21	1344.64
T_2	22.35	23.68	23.02	2485.93	2677.26	2581.59	1916.08	2157.47	2036.78
T_3	18.50	19.60	19.05	1790.65	2178.41	1984.53	1874.29	2081.05	1977.67
T_4	17.76	19.06	18.41	1634.00	1934.85	1784.42	1743.85	1952.38	1848.12
T_5	16.90	18.14	17.52	1593.40	1762.72	1678.06	1558.10	1697.35	1627.73
Mean	19.1	20.41		1951.68	2194.94		1897.84	1792.65	
CD (P = 0.05)									
Treatment (T)		0.07			0.29			18.53	
Genotype (G)		0.05			0.23			12.58	
T × G		0.10			0.41			28.39	

*Fresh weight

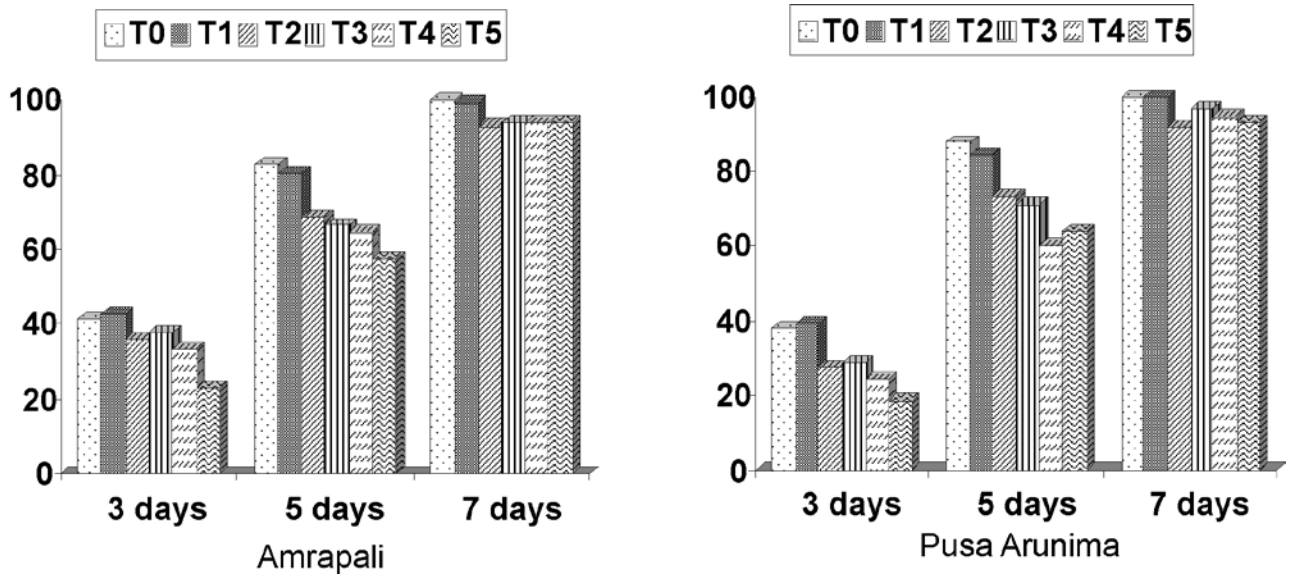

Fig. 3. Contamination (%) as affected by different pre-treatments in shoot tip culture establishment mango.

attributed to delayed senescence of treated plantlets as it is known to stimulate synthesis of cytokinin as well as having cytokinin-like activity (Binns, 2). Furthermore, it might have helped mitigating ethylene induced stress by inhibiting ethylene biosynthesis (Grossman *et al*., 11) and enhancing polyamines level in treated explants (Fletcher *et al*., 6). The *in vitro* phenol exudation in the present study was lower in explants obtained from etiolated shoots. Earlier, Sharma and Singh (18) achieved higher survival in mango shoot tip cultures. They attributed it to reduction in phenol content and polyphenol oxidase activity at pre-culture stage and *in vitro* phenol leaching by mango explants as a result of 10-day etiolation treatment of stock plants. Agitation of explants with antioxidants like ascorbic acid / citric acid or phenol adsorbent PVP further enhanced survival and slowed down the progression of necrosis.

Another interesting observation made in this experiment was a significantly reduced infection level and contaminants load on PVP treated explants. It can be assumed that since PVP was able to reduce phenol leaching efficiently (Fig. 2), it thereby, helped maintenance of high phenolics level in plant (explant) system. The higher phenolics level in PVP treated explants therefore resulted in manifestation of delayed and reduced expression of contaminants. Profound influence of explant size on culture establishment was observed. A gradual progress in explant survival was noted with the increase in explant size. The smallest explant size gave least survival. The mean value for explant survival irrespective of duration and size was significantly higher in Amrapali (85.59). Amrapali obtained highest mean value for survival (94.50) with 4.0 cm explant size, while Pusa Arunima registered the lowest survival (58.10) with 1.0 cm explants (Fig. 4). This result is in conformity with those obtained by Thomas and Ravindra (19). In addition, they observed more medium discolouration with smaller explants. The best result in the present investigation was obtained with 4.0 cm explants. Earlier, Chandra *et al.* (4) suggested use of 3.0-5.0 cm explant for culture initiation in mango for better results. Maximum survival was noted with current season shoots (3-4 month old) (58.93%), while minimum with explants procured from newly emerged flush (one-week- old) (25.90%) (Fig. 5). This is in line with earlier reports made by Thomas and Ravindra (19) and Chandra *et al.,* (4). The lower survival of one-week-old explants could be attributed to their physiological status. The newly emerged flushes in mango are coppery red due to high anthocyanin content, which contributes to total phenol content of explants. In addition, younger tissues have, in general, higher oxidative rates than older ones (Yu and Meridith, 21). Likewise, the enzyme which catalyzes the production of phenolic compounds, phenylalanine ammonia lyase (PAL), is much more active in younger tissues than in older ones (Funfgelder *et al.,* 8). Furthermore, differences in polyphenol oxidase (PPO) activity, (an important enzyme which oxidizes phenolic compounds) between tissues of different degree of maturity have been described in other crop species, with the enzyme activity decreasing with increasing physiological age (Flurkey and Jen, 7).

Furthermore, upon culturing such shoots exude higher phenol into medium (Thomas and Ravindra,

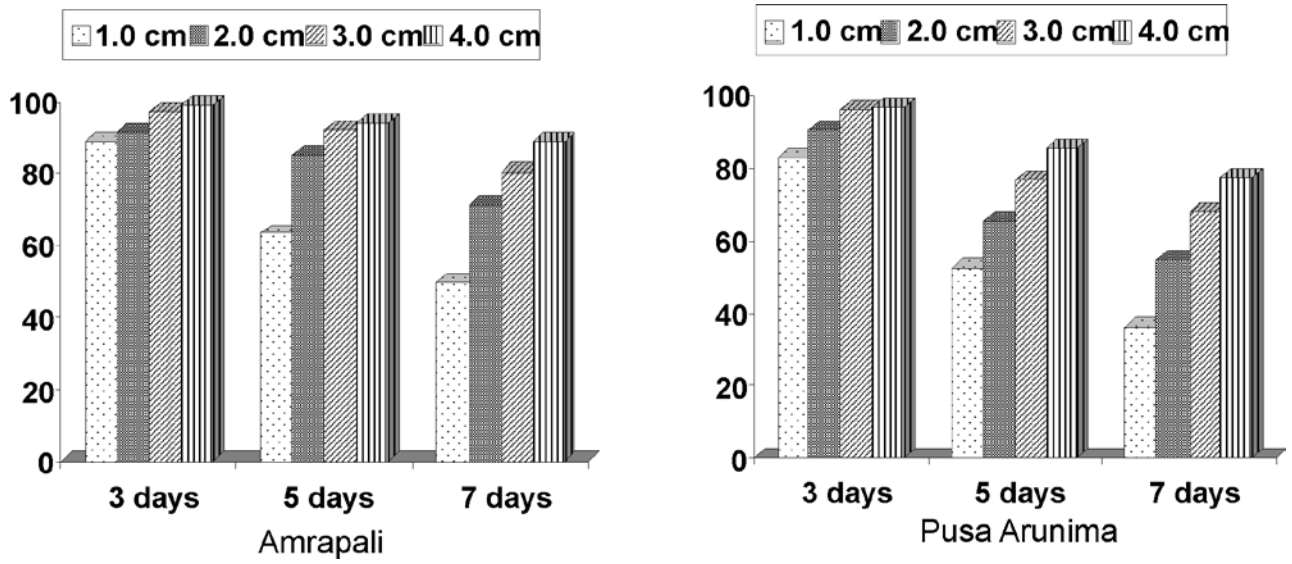

Fig. 4. *In vitro* explant survival (%) as affected by nodal segments explant size in mango genotypes.

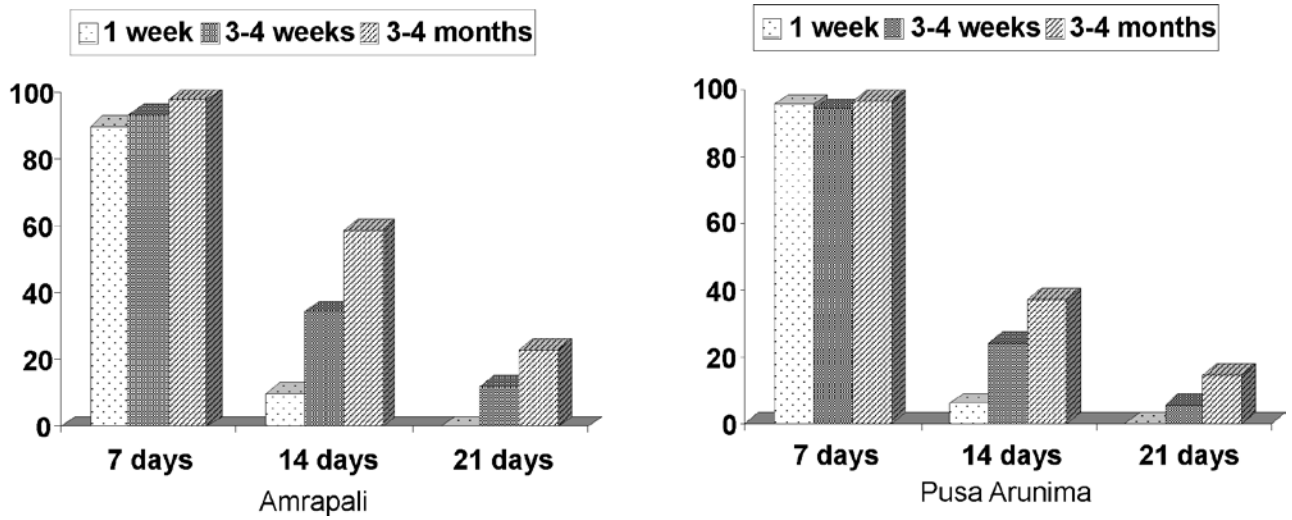

Fig. 5. Influence of age of explants on *in vitro* culture establishment (%) in monoembryonic mango genotypes.

19) and thereby, reducing explant survival and promote early expression of necrosis. Phenolic compounds are best known as auxin protectant and should be confined within plant system to stimulate growth and their release to culture medium should be reduced to alleviate the phytotoxic effects (Debergh and Read, 5).

From the data presented in Fig. 6 it was observed that explanting during March gave the best survival (62.85%), irrespective of variety and post-inoculation culture period. Variety Amrapali was found significantly superior over Pusa Arunima with respect to explant survival. Explants collected during January showed maximum contamination at all post-culture stages, while cultures initiated during March followed by September were more responsive with less microbial contaminants. Similar seasonal variations in *in vitro* culture initiation of mango shoot explants were observed by Chandra *et al.* (4). Genotype dependent *in vitro* response was observed in the present study. In general, variety Amrapali was found more responsive than Pusa Arunima. The lower survival and higher necrosis of Pusa Arunima, irrespective of pre-treatments, size of explant, age of stock plants and season of explanting might be due to higher level of phenols in this variety. As such this variety is coloured and the newly emerged

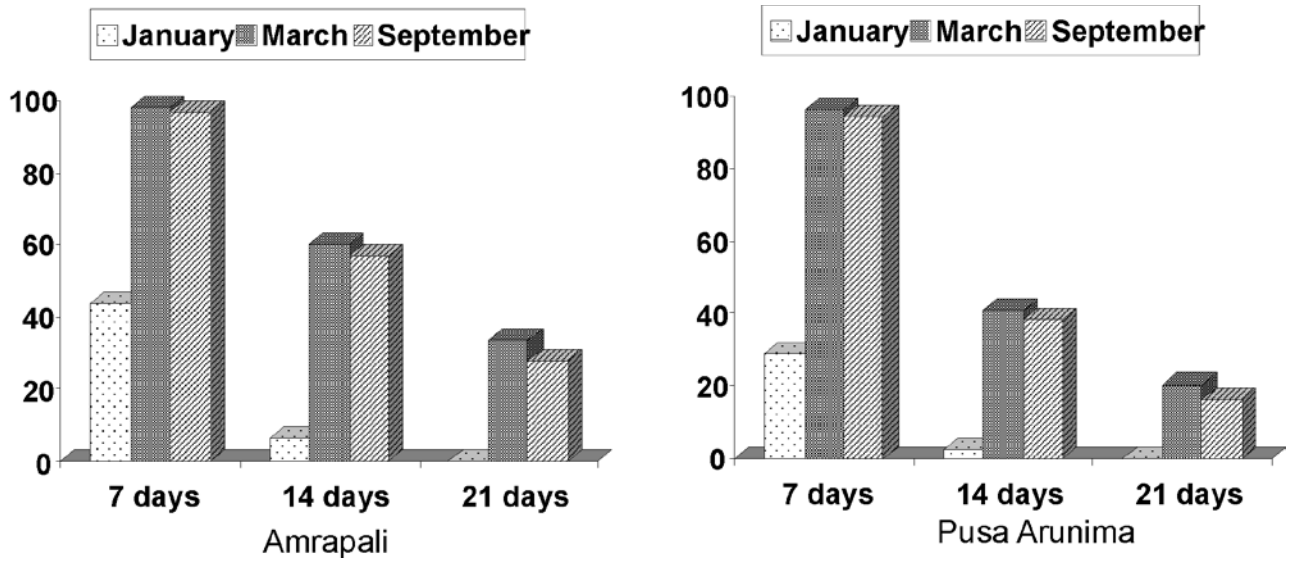

Fig. 6. Culture establishment (%) as affected by month of explanting in monoembryonic mango genotypes.

flushes in this variety are more coloured (coppery red) than Amrapali. Visual observations made for *in vitro* phenol exudation support this view. Cai and Butler (3) observed that sorghum varieties, selected for high tannin content, surrendered such large quantities of pigmented phenolics upon culturing, that the medium darkened and cultures readily become necrotic.

More explant browning was recorded on MS and its modification than B5 or its modifications (Fig. 7). Least necrosis and highest explant survival was noted on B_5(-) medium supplemented with 0.5 mg l^{-1} NAA + 2.0 mg l^{-1} BAP. Maximum necrosis and reduced survival was noted on media supplemented with 1.0 mg l^{-1} NAA + 10.0 mg l^{-1} kinetin, irrespective of genotypes. Higher salt concentration in MS medium could be a valid reason for higher browning percentage. Anderson (1) suggested that tissue browning of Rhododendron explants was accentuated by high potassium levels in MS medium. George (10) reported higher browning incidence in *Pelargonium* shoot tips cultured on medium supplemented with more than 1.0 mg l^{-1} kinetin. Likewise, addition of NAA to the *Comptonia peregrina* cultures caused the explants to turn brown (Louis and Torrey, 15).

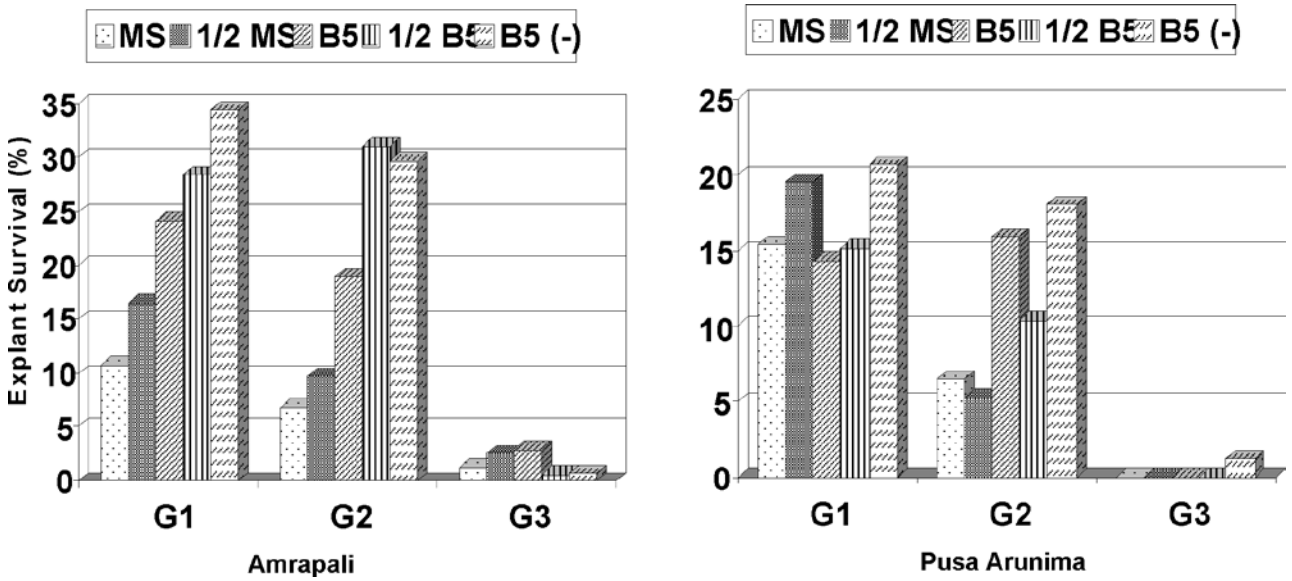

Fig. 7. Explant survival as affected by media and growth regulators in mango (21 days after inoculation).

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