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In Vitro Propagation Strategies for Elephant Foot Yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson

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Abstract

In vitro propagation method using petiole and corm explants for the cultivated species of *Amorphophallus paeoniifolius* var. *campanulatus* (Decne) Sivad. cv. Gajendra is reported. The petiole slices cultured on Murashige and Skoog (MS) medium containing 6-Benzyladenine (BA) and 1-Naphthaleneacetic acid (NAA) produced callus within four weeks of culture. Continuous maintenance of original explant along with developed callus in respective medium showed initiation of adventitious shoot buds, roots and protocorm like bodies (PLB) or corm like structures (CLS). The CLS subsequently developed into complete plants. MS medium supplemented with NAA (2.5 µM) in combination with BA (5.0 µM) was identified as the best medium for CLS induction. Histological studies of the callus revealed three developmental stages. Complete plantlets were obtained from CLS within 16-24 weeks, by maintaining the culture continuously on the same combination of initiation medium. The culturing of apical bud explants of corms on BA or Thidiazuron (TDZ) in combination with NAA resulted in the proliferation of adventitious buds. But further development was arrested. The response of corm explants to callus induction and CLS formation was very slow as compared to petiole explants. The corm explant based propagation recorded less number of CLS compared to petiole based method. Thus, plant development through CLS from petiole explants can be adopted for the large scale production of plantlets.

Key words: *Amorphophallus paeoniifolius,* corm like structures, histology, petiole explants, photoperiod

Introduction

Amorphophallus paeoniifolius (Dennst.) Nicolson var. *campanulatus* (Decne.) Sivad. (Nicolson, 1987) is a subsidiary food in the tropics (Jos et al., 1977; Prakash and Nayar, 2000). It has emerged as a commercial crop in India with good economic returns to farmers due to its high production and export potential. Because of high market demand it is being extensively cultivated in different parts of India (Misra et al., 2001). Lack of sufficient seed material of uniform size and dormancy is the major constraint limiting the production of elephant foot yam (Bhagavan et al., 2008). By adopting conventional method of propagation, multiplication rate of 1:4 and by minisett technique, multiplication rate of 1:15 could be attained (James George and Nair, 1993). Several workers reasoned that this procedure requires about 20% of each year's harvest for next season's seed and is considered a costly affair (Asokan et al., 1984). In addition to this, the method is always associated with the risk of dissemination of diseases. *In vitro* regeneration studies in this crop have yielded little success in the past (Irawati et al., 1986; Nyman et al., 1987) though in other species of this genus, it was successful. Information on *in vitro* propagation of elephant foot yam is limited due to its recalcitrant nature (Mukherjee et al., 2009).

In India, 'Gajendra' is the most preferred cultivar of *A.*

paeoniifolius (Dennst.) Nicolson. It is characterized by non-acrid, smooth corm and high yield. *In vitro* approaches in *A. paeoniifolius* would be useful to produce suitable planting materials like microcorms or plantlets in mass numbers or to induce somaclonal variants, thereby creating varibility in this vegetatively propagated crop. An attempt has been made in this paper to standardize *in vitro* propagation methods in elephant foot yam cv. Gajendra.

Materials and Methods

Effect of different explants

In vitro response of two explants viz., corm segments from mature corms and petiole segments from aseptic seedlings, were evaluated in the present study.

Corm explants were obtained from the central apical portion of the corm in *Amorphophallus* which is a condensed shoot tip covered with scale leaves having numerous axillary buds in whorls. This portion was scooped out (about 3 cm diameter and 3 cm depth) from 1 kg tuber, thoroughly washed in tap water followed by washing in dilute solution of mild detergent (1% polysorbitol, Labolene, Qualigens Fine Chemicals, Mumbai, India) for 30 min and then rinsed 2-3 times in distilled water. The preconditioned explant was dipped in ethanol (95%) and flamed, soaked in a systemic fungicide carbendazim (0.2%) (Bavistin, BASF, Mumbai, India) for an hour in a rotary shaker at 100 rpm, followed by surface sterilization in 0.1% mercuric chloride solution containing a few drops of Tween-20 for 10 min and 4-5 rinses in sterile distilled water (SDW) in the laminar-air-flow cabinet. The apical bud was then sliced longitudinally into 12-16 segments (5 mm^2) and placed on a semisolid medium prepared in culture test tubes for micropropagation. Surface sterilized explants were sliced vertically into thin segments (5 mm^2) and placed on a semisolid medium for callus induction. Murashige and Skoog (MS) medium (1962) containing 3% sucrose and 0.1% Polyvinylpyrrolidone (PVP) and varying concentrations and combinations of 6-Benzyladenine (BA), Thidiazuron (TDZ), 1-Naphthaleneaceticacid (NAA) and Gibberellic Acid *(*GA3) were used (Table 1) for micropropagation of corm explants. For callus induction, media with BA (2.5, 5, 8 µM) in combination with NAA (2.5, 5 µM) were used.

Table 1. Effect of BA and NAA on adventitious shoot bud induction using corm slices (central apical portion) in *A. paeoniifolius*

BA (µM)	NAA (µM)	Response (%)	Mean number of buds/explant
0	0.00	0.00^{f}	0.00^{c}
2	0.25	0.00^{f}	0.00^{c}
	0.5	8.30^{ef}	2.00^{bc}
	1.0	12.50^{def}	1.30^{bc}
	1.5	8.30^{ef}	3.30^{ab}
	2.5	12.50^{def}	2.00^{bc}
	5.0	4.17^{f}	3.00^{abc}
4	0.25	83.30^{a}	3.67^{ab}
	0.5	50.00^{b}	2.67^{bc}
	1.0	33.30^{c}	2.33^{bc}
	1.5	29.16^{bc}	2.67^{bc}
	2.5	58.30^{b}	3.00^{abc}
	5.0	33.30^{c}	1.70^{bc}
6	0.25	12.50^{def}	3.30^{ab}
	0.5	33.30^{c}	5.70^{a}
	1.0	15.70^{def}	2.30^{bc}
	1.5	8.30^{ef}	0.83^{bc}
	2.5	15.00^{def}	2.06^{bc}
	5.0	0.00^{f}	0.00^{c}
8	0.25	0.00^{f}	0.00^{c}
	0.5	8.30^{ef}	2.00^{bc}
	1.0	25.00^{bcd}	2.60^{bc}
	1.5	12.50^{def}	2.00^{bc}
	2.5	23.30^{bcd}	3.00^{abc}
	5.0	23.30^{bcd}	2.00^{bc}
Treatment Df (n-1)	24	15.58*	2.317*
A-C comb.(T) Df (n-1)	3	72.58*	0.956^{NS}
A-C conc. (C) Df (n-1)	5	4.114*	1.394^{NS}
T x C Df (n-1)	23	14.91*	2.055^{NS}

*Significant at $P < 0.001$ level; NS-Non-significant; Means within a column followed by the same letters are not significantly ($P < 0.05$) different as determined by DMRT

For obtaining aseptic petiole explants, artificial pollination was carried out in cv. Gajendra and mature fruits were harvested. After harvesting, the seeds were extracted, washed in running tap water, sundried for 4-5 days and used for *in vitro* germination. Seeds were thoroughly washed in tap water containing few drops of a detergent (Labolene, Qualigens Fine Chemicals, Mumbai, India) and then treated with 70% alcohol for one minute followed by few rinses in SDW. The seeds were then surface sterilized with 0.1% mercuric chloride solution containing few drops of surfactant (Tween-20) for 10 min followed by several rinses in SDW before inoculation on germination medium prepared in culture tubes with one seed per tube. MS medium supplemented with NAA (1 or 2 µM) and activated charcoal/PVP (1g l^{-1}) and 3% sucrose was used as germination medium. When the seedlings were 10-11 cm long, the young petiole was excised and segmented (2-3 mm) aseptically and cultured on semi-solid media. These explants were cultured in MS medium supplemented with varying combinations of NAA (0.0, 2.5, 5, 10 µM) and BA (0.0, 2.5, 5, 10 µM) (Table 2). To find out the best cytokinin for *in vitro* propagation, MS medium fortified with BA (2.5, 5, 10, 20 µM), TDZ (0.5, 1, 2, 4 µM) or kinetin (2.5, 5, 10, 20 µM) in combination with NAA (2.5 µM) (Table 3) was used. In all media, 3% sucrose was added. For initial culturing of petiole explants, culture tubes containing 10 ml aliquot medium was used and during further subcultures, 200 ml jam bottles containing 50 ml medium was used.

All the media were adjusted to pH 5.8, solidified with 0.7% agar and sterilized by autoclaving at 121ºC for 20 min. The cultures were transferred to fresh medium after every eight weeks. The cultures were incubated at 25 ± 1ºC in darkness for callus induction. Otherwise the cultures were maintained aseptically at 25 ± 2ºC, RH at 60% and 16:8 h light:dark photoperiod at an irradiance of 35 µmol $m^{-2}s^{-1}$ provided by cool-white fluorescent tubes.

The developed calli were maintained for 12 weeks in dark and then transferred to a 16 h photoperiod with an irradiance of 35 µmol $m^{-2}s^{-1}$ supplied by cool-white fluorescent lamps (Philips, India) for plant regeneration.

Data on callus induction was recorded after 12 weeks of incubation. Approximately 0.5 cm^2 sized callus mass (approximately100-150 mg) was used for subculture trials. Organogenesis in terms of root or shoot development and entire plantlet formation was recorded after 16th week of subculture.

Effect of photoperiod on callus induction and organogenesis (petiole derived callus)

Effect of different photoperiods (0, 16 or 24 h) at an irradiance of 35 µmol $m^{-2}s^{-1}$ on callus induction and organogenesis were studied on MS medium containing NAA (2.5 µM) and BA (5 µM) (identified as optimal medium). In another experiment, the callus raised in MS medium supplemented with NAA (2.5 µM) + BA (2.5 µM) and NAA (2.5 µM) + BA (5 µM), after eight weeks of continuous incubation in dark was subjected to two treatments - continuous dark treatment vs. 16 h light for 12, 16 and 24 weeks, to study the development of protocorm like bodies (PLB) or CLS.

Hardening of *in vitro* regenerants

In vitro raised plantlets were subjected to hardening by sequential culturing on paper bridge placed in half-strength MS liquid medium containing 1.5% sucrose initially for two weeks and then transferred to sucrose free half-strength MS medium for another two weeks. Plantlets thus obtained were potted in plastic pots containing sand and vermiculite (1:1) and covered with polyethylene bags to maintain high humidity. Relative humidity was reduced gradually by increasing the number of perforations on the bags. Two weeks after potting, bags were completely removed.

Histological examinations

Calli at different stages of growth were fixed in FAA (formaldehyde:ethanol:acetic acid 1:18:1) and dehydrated in a xylene–alcohol series and then the material was embedded in paraffin wax (58-60ºC). The embedded material was sectioned (10 µM) using a rotary microtome and stained with toluidine blue (O'Brien et al., 1964) after subjecting to a dewaxing process (Johansen, 1940).

Experimental design and data analysis

All experiments were conducted in a Randomized Block Design (RBD) and each treatment consisted of three replications with 10-15 cultures per replication. Data was analyzed statistically by analysis of variance (ANOVA) and differences contrasted ($P < 0.05$) using Duncan's Multiple Range Test (DMRT).

Results and Discussion

Effect of explants

Corm explants

The expansion of explant was the initial response in the medium supplemented with growth regulators. Explants cultured on hormone free MS medium and media supplemented with either BA or TDZ did not induce adventitious shoot buds. BA (0.5 µM) in combination with TDZ (0.5 µM) induced multiple shoot buds (4.67 shoot buds). The stimulatory effects of TDZ on bud breaking and shoot regeneration has been reported (Husain et al., 2007). However, further growth of shoot buds was suppressed and cultures deteriorated soon, which is in tune the with other reports (Preece and Imel, 1991; Kondamudi et al., 2010).

A combination of BA and NAA was found to be the most efficient in inducing maximum healthy shoot buds (83%). BA (4 µM) in combination with NAA (0.25 µM) produced an average of 3.67 buds/explants (Table 1). ANOVA revealed a significant ($P < 0.001$) interaction between auxin-cytokinin combination as well as concentration in inducing explant response. However, regarding mean number of buds per culture, there was no significant interaction between auxin and cytokinin. Within six weeks of incubation, an average of 5.7 shoot buds per culture was initiated on MS medium supplemented with BA (6 µM) in combination with NAA (0.5 µM) (Fig.1). The buds were separated and placed in varying levels of BA (0.05-8 µM), NAA (0.5-5 µM) and GA3 (01-1µM) supplemented medium for elongation and further development. Though there was initial vigour in response, the buds soon turned brown and further growth ceased. Cultures raised on MS medium supplemented with BA (8 µM) and NAA (2.5 µM) when incubated continuously in dark showed development of nodular calli.

In the callus induction medium, the corm explants expanded initially and turned black after 4-5 weeks of incubation in MS medium supplemented with varying levels of BA (2.5-8 µM) and NAA (2.5-5 µM). The blackened explants, upon further transfer to same hormonal combination of medium resulted in the development of pinkish calli within 20-24 weeks which on further subculture turned into a blackish hard compact mass. Callus was induced in all the media containing BA and NAA in the dark except hormone free media. The calli revealed two types of organogenic response after subculture i.e., root development and protocorm like bodies (PLB) or corm like structures (CLS). Organogenesis in the form of roots was observed in most of the callus cultures. Shoot formation however, did not occur from the callus induced from corm explants directly. Alternatively, complete plantlets were obtained via CLS, where, simultaneous development of root and shoot was observed after 36 weeks of culture.

A long term retention of explant in hormone fortified media was required for the induction of CLS and subsequent plantlet formation. The highest frequency (62.5%) of plantlet development through CLS was recorded in MS medium supplemented with 5 µM BA and 2.5 µM NAA. Similar results were observed in *A. campanulatus* var. *hortensis*, where, formation of globular structures due to long term retention of corm callus was reported (Irawati et al., 1986). These globular structures subsequently produced shoots and roots.

Petiole explants

The petiole segments obtained from the germinated seedlings (Fig. 2), when cultured on growth regulator supplemented medium, showed development of friable, pale callus from the basal cut surface after four weeks of inoculation. The callus was induced in all the media except M1 (hormone-free medium) and media devoid of NAA (M2 and M3). After 12 weeks of culture, calli grew into large masses with an area of 1.5-2.0 cm^2 At 12^{th} week, the frequency of callus induction was 100% in M6, M7, M9, M11 and M15 combinations (Table 2). The induced calli were soft, pinkish white, powdery and fragile (Fig. 3) in nature. Primary calli on subculture to respective media at monthly intervals, turned into a compact, nodular, white mass of cells which later turned into a blackish or greenish hard structure. Complete plantlets were also obtained from the original calli via CLS (Fig. 4). The 16 week old calli on the respective media revealed three types of organogenic response i.e., root development, adventitious shoot buds and protocorm like bodies (PLB) or corm like structures (CLS) (Fig. 5). The CLS subsequently developed into complete plants.

The frequency of cultures showing adventitious shoot bud formation was maximum (83.3%) in M7 medium

Fig.1. Development of axillary shoot buds (x1.2) from corm explants
Fig.2. Seed germination and multiple embryo formation of *A. paeoniifolius* on agar gelled MS medium
Fig.3. Formation of pinkish callus from petiole explants
Fig.4. Formation of plantlet from petiole derived hard compact callus along with original petiole explant
Fig.5. Formation of shoot buds (black arrows) and CLS resulting in complete plantlets from subcultured callus
Fig.6. Formation of rooted shoot via CLS from petiole derived callus
Fig.7. Hardening of *in vitro* raised plantlets in plastic pots

(Table 2, Fig. 5), whereas, the number of shoot buds recorded in M6 medium was significantly higher (8.7 buds/culture) than all the other combinations. The frequency of CLS formation was significantly ($P < 0.05$) high in M7 (93.3%) medium followed by M8 (90%) and M6 (83.3%). The number of CLS or rooted plantlet development after 16th week was maximum in M6 (4.8 per culture) followed by M7 (4.4 per culture) and M8 (3.9 per culture). Auxin and cytokinin had significant ($P < 0.001$) interactive effect on both adventitious shoot bud and CLS formation (Table 2). In both M6 and M7 medium, shoot buds and CLS were formed in the same culture. Further subculturing of the calli in the respective medium resulted in continuous proliferation of CLS. During the course of subculture, most adventitious buds grew slowly followed by a suppression of growth at a later stage. *In vitro* developed CLS showed potential to produce complete plants with simultaneous development of root and shoot system on the same media (Fig. 6). In the petiole derived calli, numerous adventitious buds were formed on different combinations, but the conversion into PLB or CLS only led to the formation of complete plantlets. Protocorm like bodies that are able to differentiate into shoots and roots (da Silva et al., 2000) resemble somatic embryos, however, the shoots and roots of regenerated plantlets from PLBs were not on the same axis. Thus, there are two schools of thoughts regarding the identity of PLBs; one suggests that PLBs are somatic embryos (Ishii et al., 1998; Chen et al., 2000) and the others assume that PLBs differ from somatic embryos (Cui et al., 2008; Tian et al., 2008). Protocorm like bodies have been identified in a wide range of other plant genera including *Anthurium* (Yu et al., 2009), *Colocasia* (Abo El-Nil and Zettler, 1976), *Musa* (Venkatachalam et al., 2006), *Pinellia* (Liu et al., 2009), and *Syngonium* (Cui et al., 2008). In *Amorphophallus*, PLBs are called corm like structures (CLS) (Hu et al., 2008).

It was observed that all the shoots developed through CLS pathway upon reaching 1-2 cm length, developed roots

Table 2. Effect of BA and NAA on inducton of PLBs and adventitious buds from petiole derived callus

Treatment	Plant growth regulators (µM)		Explants inducing PLB at 16 weeks of culture (%)[a]	Explants inducing shoot buds at 16 weeks of culture (%)[a]
	NAA	BA		
M1	0	0	0.00[d]	0.00[d]
M2	0	2.5	0.00[d]	0.00[d]
M3	0	5	0.00[d]	0.00[d]
M4	0	10	10.83 ± 0.83[d]	14.1 ± 3.0[ef]
M5	2.5	0	35.75 ± 4.24[c]	35.75 ± 4.24[de]
M6	2.5	2.5	90.33 ± 9.6[a]	72.7 ± 13.6[ab]
M7	2.5	5	93.3 ± 3.3[a]	83.3 ± 8.8[a]
M8	2.5	10	90.0 ± 1.73[a]	70.0 ± 15.27[ab]
M9	5	0	33.3 ± 12.01[b]	43.3 ± 3.3[cd]
M10	5	2.5	68.8 ± 1.13[b]	64.4 ± 8.02[abc]
M11	5	5	62.2 ± 6.17[b]	62.2 ± 6.17 [abc]
M12	5	10	60.6 ± 6.35[b]	73.3 ± 12.01[ab]
M13	10	0	0.00[d]	0.00[f]
M14	10	2.5	0.00[d]	0.00[f]
M15	10	5	50.0 ± 5.7[bc]	50.0 ± 5.7[bcd]
M16	10	10	53.3 ± 3.3[b]	60.0 ± 10.0[bc]
Treatment Df (n-1)		15	51.91***	17.76***
Auxin conc. (A) Df (n-1)		3	190.53***	59.81***
Cytokinin conc. (C) Df (n-1)		3	28.40***	16.91***
A x C Df (n-1)		15	51.91***	17.76***

[a]Means within a column followed by same letters are not significantly different as determined by DMRT ($P < 0.05$); ***Significant at $P < 0.001$ level

and thus complete plants were formed on the same medium irrespective of the media combination. Adventitious shoot buds developed in this medium however, failed to sustain further growth to form complete plants. ANOVA revealed significant ($P < 0.001$) interaction of the type of cytokinin and concentration in inducing callus and morphogenesis (Table 2). MS medium supplemented with NAA (2.5 µM) in combination with BA (5.0 µM) was identified as the best medium for CLS production. BA (2.5 µM) in combination with NAA (2.5 µM) was one of the best combinations in terms of percentage of shoot bud and CLS induction and number of shoot buds as well as formation of complete plants. The combination of BA (5 µM) and NAA (5 µM) also showed response to a lesser extent. However, higher concentration of BA (10 µM) and NAA (10 µM) caused significant reduction in the formation of CLS. In contrast, Hu and Li (2008) reported inefficiency of equimolar concentration of NAA and BA to induce morphogenetic response in *A. albus*. In medium M2 and M3, where BA alone was added, shoot bud formation and CLS formation were absent. When high concentration of BA (10 µM) was added (M4), shoot buds and CLS was induced at a very low frequency (Table 2). A report substantiating this observation is available in *A. albus* (Hu and Li, 2008). Hu et al. (2008) reported that a molar ratio of cytokinin to auxin (4:1) was a key factor in promoting high frequency CLS formation in *A. albus*, but in the present study such a rigorous ratio was not observed. Maximum frequency of CLS formation in the cytokinin to auxin ratio of 1:1 (M6) or 2:1 (M7) in addition to 4:1 (M8)

was observed in the present morphogenetic study on *A. paeoniifolius*. A moderate concentration of BA and low concentration of NAA favoured CLS formation in the present study, which is in agreement with the report on *in vitro* studies on *A. konjac* (Liu et al., 2001).

The suppression of adventitious buds observed in *A. paeoniifolius* cultures is comparable to previous reports on *A. albus* (Hu and Li, 2008) and *A. rivieri* (Hu et al., 2005). The formation of CLS and adventitious buds in the same culture indicates that possibly shoot buds were converted into CLS or else shoot bud initials and CLS initials developed simultaneously from the callus under special conditions based on the concentration of NAA and BA incorporated in the medium.

Of the three cytokinins (TDZ, BA or Kin) tested, BA showed superiority over other cytokinins and was significant ($P > 0.05$) on callus and CLS development (Table 3). Among all the cytokinins, kinetin was the least effective one and is in agreement with some previous reports (Huang and Lu, 1993; Kondamudi et al., 2010). The superior activity of BA has been reported in a wide range of species including the family Araceae (Komalavalli and Rao, 2000; John et al., 2001).

Effect of photoperiod on callus induction and morphogenesis

Amongst the three photoperiods tested, highest frequency of callus induction (91.3%) was recorded in cultures incubated in continuous dark (0 h). Callus formation did not occur in the other two photoperiods viz., 16 h light:8 h dark and 24 h light. Exposure of explants to irradiance (35 µmol $m^{-2}s^{-1}$) for 16 h or 24 h resulted in gradual colour change of explant from green to brown, whereas, the callus developed in the dark, upon transfer to three photoperiods (0, 16, 24 h) resulted in organogenesis in terms of CLS formation. Callus cultures exposed to continuous light produced a low percentage (30%) of CLS. In *A. albus*, *in vitro* corm formation was reported to be insensitive to photoperiods (Hu et al., 2006) as against *A. paeoniifolius* where, photoperiod has a role in *in vitro* corm formation. In wheat and in a weedy grass *Echinochloa colona*, incubation of callus in dark followed by light period was conducive for somatic embryogenesis and incubation in continuous light inhibited somatic embryos (Samantaray et al., 1997).

Effect of continuous dark and light incubation on CLS induction from callus

The callus initiated in dark and further maintained in dark for 12-24 weeks, had significantly ($P < 0.05$) high frequency of globular structures. However, the frequencies of CLS formation were similar after 16 weeks in both treatments (Fig. 8). At 12th and 16 th week the differences in CLS induction % was significant at $P < 0.05$. At 24th week there was no significant difference in the percentage of PLB induction between the cultures receiving light and dark treatment ($P < 0.05$) using DMRT. Dark treatment for newly inoculated explants can enhance morphogenesis. The present finding is in conformity with morphogenetic studies on apple (Welander, 1988) where, maintaining the leaf segments of apple in the dark for the first three weeks of culture enhanced the subsequent regeneration of adventitious shoots and embryo-like structures. Similarly, an initial period of dark culture increased somatic embryogenesis in *Triticum aestivum* (Ozias-Akins and Vasil, 1983) and water melon (Compton, 1999). Studies on regeneration potential of callus by dark pre-conditioning is available in many crops (Punja et al., 1990; Leblay et al., 1991; Mohamed et al., 1992).

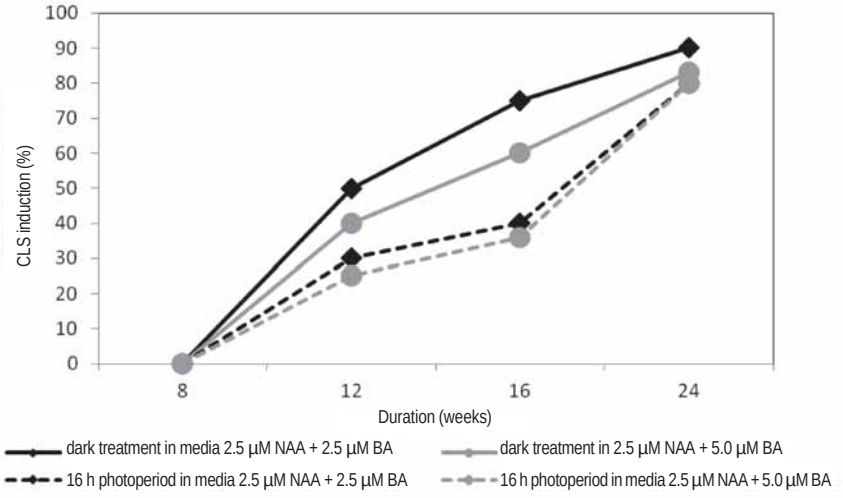

Fig. 8. *In vitro* development of CLS from callus induced in dark for initial eight weeks and then dark (24 h) and light (16 h) (35 µmol $m^{-2}s^{-1}$) treatment

Table 3. Effect of cytokinins on induction of callus and CLS from petiole explants

Cytokinins (µM) with NAA (2.5 µM)			Explants producing callus at 12 weeks of culture (%)[a]	Explants inducing CLS at 16 weeks of culture (%)[a]
BA	TDZ	Kin		
-	-	-	00.0^{i}	0.0^{e}
-	0.5	-	53.3^{ef}	6.6^{de}
-	1.1	-	36.6^{g}	36.6^{b}
-	2.2	-	20.0^{h}	3.3^{de}
-	4.4	-	00.0^{i}	0.0^{ef}
2.5	-	-	90.0^{a}	80.0^{a}
5	-	-	86.6^{ab}	76.6^{a}
10	-	-	76.6^{bc}	13.3^{cd}
20	-	-	70.0^{cd}	10.0^{de}
-	-	2.5	63.3^{de}	16.6^{cd}
-	-	5	53.3^{ef}	10.0^{de}
-	-	10	43.3^{fg}	23.3^{c}
-	-	20	23.3^{h}	3.3^{d}
Treatment Df (n-1)		12	53.642***	43.183***
Cytokinin type(T) Df (n-1)		2	158.6***	76.2***
Cytokinin concn(C) Df (n-1)		3	42.98***	48.4***
T x C Df (n-1)		11	42.05***	41.018***

[a]Means within a column followed by same letters are not significantly different as determined by DMRT ($P < 0.05$); ***Significant at $P < 0.001$ level

Hardening of *in vitro* raised plants

The complete plantlets when passed through *in vitro* as well as *ex vitro* hardening stages (Fig.7) showed a 50% survival. In *Amorphophallus*, 70-80% mortality was reported in the hardening and field establishment of *in vitro* raised plants (A. Mukherjee, Regional Centre, Central Tuber Crops Research Institute, Bhubaneswar, Odisha, India, personal communication).

Histological analysis of regeneration process

The histology of callus at the initial stage revealed the presence of numerous scattered vascular nodules (Fig. 9a) along with starch cells and tannin or mucilage cells. Raphides were also observed in the callus. The nodules, meristemoids or growth centers were localized clusters of cambium like cells which could become vascularized due to the appearance of trachiedal cells (Fig. 9b) in the centre. Meristemoid cells were compact and small exhibiting densely stained cytoplasm (Fig. 9c) compared to surrounding non-meristemoid cells that were large and loosely packed. Meristemoids were found to originate from the peripheral region of organogenic callus cells. Histological preparations revealed the origin of shoot primordia to occur both embedded in the inner tissues of organogenic calli (Fig. 9c) and also at the superficial layer (Fig. 9f black arrow). The proportion of meristemoid formation depended especially upon the type and age of the explant and the type of morphogenic callus induction medium used during regeneration.

Formation of vascular nodules in callus cultures as revealed in the histological examinations was associated with an early stage of the development of shoot meristems (Chen and Galston, 1967; Kulchetscki et al., 1995). Hu et al. (2005) reported that in *A. rivieri*, only meristemoids formed in the superficial layers could develop into plants, while those derived in the inner layers of the callus mostly developed into abnormal adventitious buds or showed retarded growth without

further development, which might be due to the structural restriction from the peripheral cells. Superficial cell layers were considered as the origin of forming adventitious buds (Barker and Steward, 1962; Budimir, 2003). The meristemoids formed in the subepidermal cells were known to produce PLBs (Kaur and Bhutani, 2010). According to Lee and Lee (2003) the globular mass of cells accumulate starch grains and protoderm formation takes place and thus gets differentiated into a CLS.

Besides, root primordia (Fig. 9d) were visible in the calli studied. Globular mass of meristematic cells surrounded by a protoderm (Fig. 9e, 9g) which probably developed into CLS having potential to develop into complete plantlet with a clear root-pole and shoot-pole (Fig. 9h) was also observed. The structure at shoot pole and root pole development stage showed resemblance to a somatic embryo, but the presence of periderm, cortex and pith in the longitudinal section, its deep seated nature (Fig. 9h) and vascular connection with the mother explant revealed its origin as a CLS or PLB (Fig. 9f). Simultaneous occurrence of both shoot bud (black arrow) and CLS (red arrow) were also observed (Fig. 9f).

Histological studies on PLB clearly showed vascular connection with the maternal explants and lack of bipolar structure, thus attributing its difference from somatic embryos. A somatic embryo is an independent entity with no vascular connection between the somatic embryos and parental explants (Chengalrayan et al., 1997). For the development of a somatic embryo, a single embryogenic cell undergoes a series of transverse

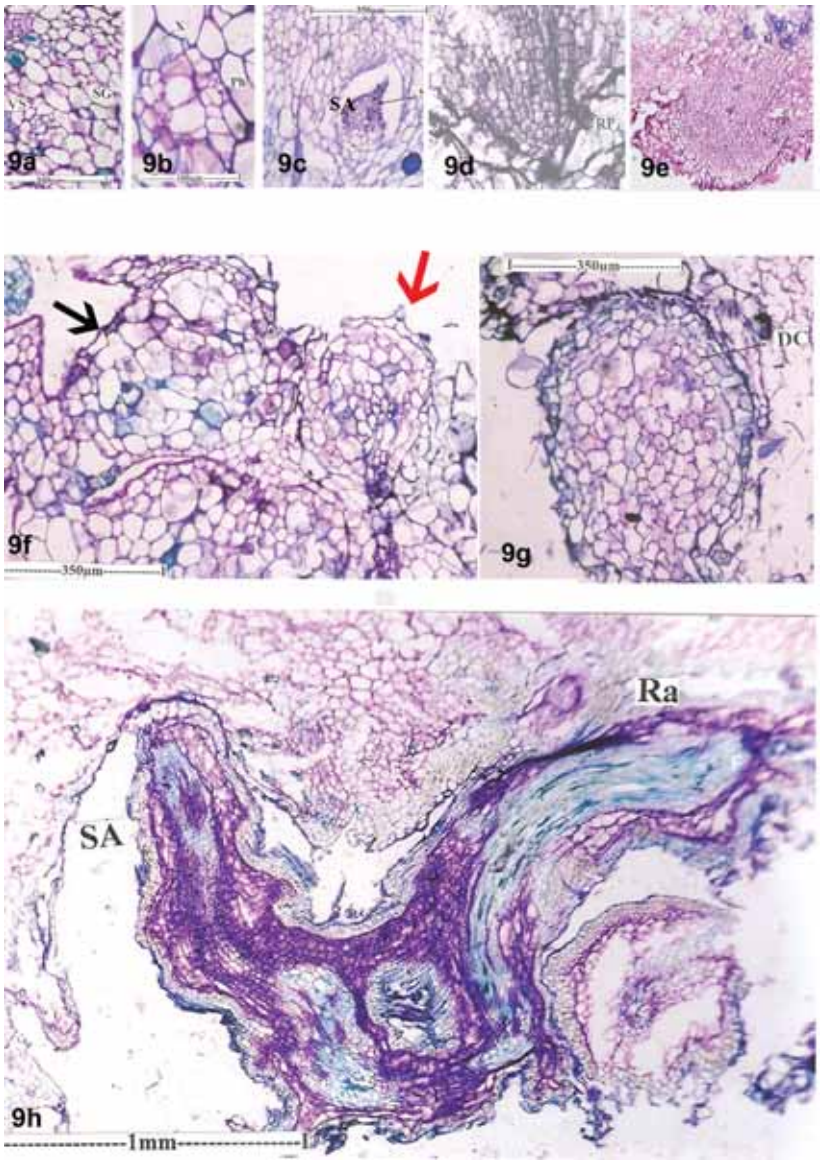

Fig.9a. Formation of vascular strands (VS), starch grains (SG) and mucilage channels (green coloured) in the callus
Fig.9b. Tracheid (xylem –X) and phloem (Ph) in the callus
Fig.9c. Formation of shoot buds in inner most layer which may hinder their further development
Fig.9d. Root apex
Fig.9e. Globular mass of cells surrounded by periderm showing formation of CLS
Fig.9f. Development of shoot buds (black arrow) and CLS (red arrow) on the same callus mass
Fig.9g. Another CLS surrounded by periderm
Fig.9h. A deep seated CLS showing both root and shoot pole

and longitudinal divisions, passing through globular, torpedo and cotyledonary stages for dicots or globular, scutellar and coleoptilar stages for monocots. The germination of somatic embryo is analogous to seed germination, resulting in shoots and roots on a bipolar structure. However,

PLBs or CLS morphologically resemble somatic embryos. Depending on plant species and explant types, many PLBs can be produced per explant and PLBs can produce shoots and roots directly (Cui et al., 2008; Tian et al., 2008).

Frequent occurrence of a small amount (< 10%) of albinos or variegated phenotypes observed in the present study may be due to the long term *in vitro* incubation of cultures and lesser frequency of subculture, which may affect callus growth, plantlet formation and development (Nyman et al., 1983; Irawati et al., 1986). Another possibility would be due to the pre-existing somatic variation. Continuous vegetative propagation of highly heterozygous plants may allow an increase in variation through the accumulation of somatic mutations because of the missing meiotic sieve (Buss, 1983; Zohary, 2004). *In vitro* regeneration differs from traditional vegetative propagation by allowing single or few cells from a small segment of explants to differentiate *in vitro* and develop into plantlets, which provides a greater chance of uncovering mutated cells. Hence, *in vitro* techniques can be a method for induction of variability in cultivars, especially as the cultivated species are losing their ability to reproduce sexually because of selective pressures to preferentially allocate more photosynthate towards tuber production (Singh and Gadgil, 1995) and extreme protogyny (Arakeri, 1956; Sreekumari, 2000).

This is the first report on *in vitro* multiplication of *A. paeoniifolius* from petiole explants. *In vitro* culture techniques for other crop species in the family Araceae have been found useful for propagation and elimination of various pathogens (Hartman, 1974; Balogun, 2009). *In vitro* propagation techniques can be advantageous over conventional methods if no significant number of somaclonal variants result (Asokan et al., 1984). Moreover, just like microtuber in potato (Gopal et al., 1998) or cormlet in *Gladiolus* (Dantu and Bhojwani, 1995), CLS could be treated as microcorms or seed corms which can be used in germplasm storage and transportation (Hu and Li, 2008).

The morphogenetic pathway evolved in the present study requires 16-24 weeks for whole plant development starting from petiole explant, initiation of callus followed by CLS formation. This makes the entire procedure technically simple and easy to adopt at a commercial level. The response of corm explants to callus induction and CLS formation was very slow as compared to petiole explants. The number of CLS or plantlets were also very less in corm derived callus compared to petiole explants. Such recalcitrant nature of corm explants have been reported in *A. campanulatus* var. *hortensis*, where it took 24-36 weeks for the development of plantlets (Irawati et al., 1986).

In conclusion, plantlet production through CLS from petiole-derived callus could be an independent pathway in plant regeneration for large-scale production of plantlets, thus saving corm tissue, ensuring rapid multiplication of the most accepted cultivar of the crop.

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