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Preparation and Low Temperature Short-term Storage for Synthetic Seeds of *Caladium bicolor*

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Abstract

An efficient somatic embryo encapsulation and *in vitro* plant regeneration technique were established with *Caladium bicolor,* an important ornamental plant. Tuber derived embryogenic callus (95.50%) was obtained on Murashige and Skoog (MS) medium amended with 0.5 mg L^{-1} α-Naphthalene acetic acid (NAA) + 0.5 mg L^{-1} 6-Benzyladenine (BA). The embryogenic callus later differentiated into somatic embryos in the same plant growth regulators (PGRs) added medium (NAA and BA). The induced embryos matured and developed into plantlets in NAA and BA added media; maximum plantlets development was observed at 1.0 mg L^{-1} NAA + 1.0 mg L^{-1} BA supplemented medium. Synthetic seeds were produced by encapsulating embryos in gel containing 3.0% sucrose + 3.0% sodium alginate and 100 mM of calcium chloride. The highest synthetic seed germination (97.6%) was observed on medium supplemented with 1.0 mg L^{-1} NAA + 1.0 mg L^{-1} BA. The synthetic seeds were kept at low temperatures for storage; the encapsulated beads were viable and demonstrated good germination even after 12 weeks of storage at 4 °C. The plantlet recovery frequency was however declined with time. The synthetic seed derived plantlets were morphologically similar to the mother plant.

Keywords: encapsulation, low temperature storage, ornamental specie, plant regeneration, somatic embryos

Introduction

Ornamental plants constitute an important component in floriculture industry (Ali *et al.,* 2007). It includes bulbous, tuberous, foliage plants, used mostly as indoor decorative, landscape and potted plants. *Caladium* is one important genus in tuberous, araceous group of ornamentals. The *Caladium*'s leaves are colourful, often have a mix of green and white or red, white with red blotches, green veins and some have lavender spots (Deng *et al.,* 2006). The plant also shows a wide range of leaf shape variation and is considered to be a favourable ornamental aroid (Ahmed *et al.,* 2002; 2004). The araceous plants including *Caladium* are grown primarily in New World Tropics (Bown, 1988). The genus *Caladium* contains about 12 species and over 2000 cultivars (Wilfret, 1993). *Caladium* is propagated vegetatively, which is a slow process. As an alternative, multiplication is currently being accomplished by tissue culture and successful faster propagation was achieved using various tissues (Zhu *et al.,* 1993). The plant can also be propagated by seeds, but seedling variation is one of the limitation factors in populations; in asexually propagated ornamentals, hybridization has not often been practised as these plants demonstrate sexual and physiological incompatibilities (Repellin *et al.,* 2001). The need of improving genetic base and the conservation of *Caladium* cultivars has been realized lately, as the araceous genera are susceptible to a number of viruses (Rivas *et al.,* 2005). Recently, concerns have also been raised about the drastic decline in the number of cultivars and the loss of genetic diversity due to other socio-economic reasons in the last century (Deng *et al.,* 2007). Currently, molecular and biotechnological methods have been applied to ornamentals in order to add and alter genetic traits like alteration of plant morphology, flower size with long vase-life, persistence of the fragrance, modification of floral pigment, disease and pest resistance etc. (Wilfret, 1993). In order to exploit biotechnological methods an efficient plant regeneration system is however essential, that could generate true to type plants and induce transgenic effects in masses. In one effort, maize anthocyanin regulatory gene *Lc* was inserted into an albino cultivar by *Agrobacterium*-mediated genetic transformation that synthesized over production of anthocyanin in *C. bicolor* leaves (Li *et al.,* 2005).

Beside improvement, the erosion of genetic diversity can also be prevented by mass multiplication and simultaneous conservation of germplasm both by *in situ* and *in vitro* methods. The encapsulation technology has received considerable attention in recent time as a potential conservation propagation system (Rai *et al.,* 2009). The technique not only reduces the cost of micropropagated plants, it has also been employed for short to long-term storage of germplasm with exchange possibility (Danso and Ford-Lloyd, 2003). Synthetic seeds (artificial or somatic seeds) are analogous to the true or botanical seeds, and consist of a somatic embryo surrounded by one or more artificial layers forming a capsule (Cangahuala-Inocente *et al.,* 2007). The synthetic seeds have multiple advantages including uniformity in production, higher scale-up capacity, potential for automation production

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process, easy handling and transportation and potential long-term storage of elite germplasm (Singh *et al.,* 2007).

In this present communication, the processes of encapsulation of somatic embryos as well as other plant parts and *in vitro* storage of synthetic seeds of *C. bicolor* were described. The objective of this study was to develop a protocol for synthetic seed production in *C. bicolor* and short-term *in vitro* storage of encapsulated embryos. The study assessed 1) the suitable sowing medium for synthetic seed germination under the influence of various PGRs and 2) the short-term storage of encapsulated explants at cold (0 and 4 °C) temperatures and the germination ability after storage.

Material and methods

Establishment of callus, embryogenic tissue and embryos

The tubers of *Caladium bicolor* Vent. cv. 'Bleeding Heart', collected from Jamia Hamdard herbal garden was used as experimental material. A preliminary washing under running tap water for 30 min was made in order to reduce the microbial flora to a substantial extent. The tubers were sterilized with cetrimide (a detergent) and rinsed in sterile double-distilled water. These were dissected into 3-4 mm long pieces before inoculation. Later, 70% ethanol was added, kept for 1-2 min and rinsed with sterilized double distilled water. The pieces of tubers were finally sterilized by immersing the pieces in 0.1% mercuric chloride (sterilizing agent) for 2 min. The tuber pieces were placed on Murashige and Skoog (MS), supplemented with 30 g l^{-1} sucrose and 100 mg l^{-1} myo-inositol. For callus induction, different concentrations of 2,4-Dichlorophenoxyaceticacid (2,4D) 0.25, 0.50 and 1.0 mg L^{-1}, α-Naphthalene acetic acid (NAA) 0.25, 0.50 and 1.0 mg L^{-1} and 6-Benzyladenine (BA) 0.25, 0.50 and 1.0 mg L^{-1} were used, respectively. The presence or absence of early stages of somatic embryos was characterized by small, dense ovular or nearly globular structures, which were clearly visible with or without magnifying lenses.

The efficiency of plant growth regulators (PGRs) and their concentrations were monitored by observing callus and formation of embryos. The embryogenic sectors and other cultures were routinely sub-cultured at an interval of 4 weeks. The embryogenesis percentage and somatic embryo number/callus mass were periodically recorded.

Encapsulation of explants

Somatic embryos of *C. bicolor* were encapsulated in different concentrations (2.0%, 2.5%, 3.0% and 3.5%, w/v) of sodium alginate and calcium chloride, $CaCl_2.2H_2O$, using 75, 100 and 125 mM. The sodium alginate solution was prepared by adding 3.0% sucrose and different concentrations of sodium alginate in double distilled H_2O, and later sterilized at 121 °C. The tissues were mixed with sodium alginate for a few seconds, picked up by pipette and placed in sterilized aqueous solution of calcium chloride for varying times (5, 10, 15 and 20 min) for hardening. With occasional agitation on a rotary shaker, it resulted in bead formation. The beads were taken out by decanting off the calcium chloride solution and washed with sterilized water. The freshly prepared beads were transferred to MS, fortified with different concentrations of PGRs. The whole procedure was carried out under strict aseptic conditions. The embryogenic callus, leaf, petiole and root were also attempted for encapsulation. The beads (other than encapsulated somatic embryo) were cultured on medium to evaluate the viability and response of the encapsulated explants.

Cultural medium condition for development of plantlet from synthetic seeds

The encapsulated beads were cultured directly on MS, amended with 0.50 mg L^{-1} BA. Other PGR (mg L^{-1}) combinations i.e. 0.12 NAA + 0.12 BA, 0.25 NAA + 0.25 BA, 0.50 NAA + 0.50 BA, 1.0 NAA + 1.0 BA and 1.5 NAA + 1.5 BA were also used to test the conversion efficiency of encapsulated embryos. For plant development purpose, the beads were maintained under culture room conditions at 100 $\mu mol^{-2}s^{-1}$ light intensity, 25±2 °C temperatures and 70-80% relative humidity with 16/8 h day/night photoperiod.

Storage of capsule at different temperatures

To assess the short to medium term storage, the synthetic seeds were cultured in 90 x 15 mm Petriplates without any medium (sealed with parafilm) and were kept at 0 and 4 °C for various periods (6, 9 and 12 weeks). After low temperature storage at various periods, the synthetic seeds were taken out and cultured on MS amended with optimized NAA and BA for plantlet development.

Statistical analysis

The data on the effects of PGRs on synthetic seed in developing plantlet with or without storage were analyzed by one-way analysis of variance (ANOVA), followed by least significant difference (LSD). Values are means of three replicates from two experiments with six synthetic seeds in each replicate. The presented mean values were separated using LSD at $p \leq 0.05$.

Results and discussions

Embryogenic culture, embryo formation and plant regeneration

Tubers were used as explant for the establishment of callus for which the MS medium was amended with various PGRs (Table 1). Among all the PGRs tested, the combination of 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BA showed maximum callus induction frequency (95.50%), followed by 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BA treatment; 2,4-D also induced callus at a lower rate (data not presented). Initially, the callus was creamy in color (Fig. 1a), later transformed into green embryogenic

Table 1. Effect of various NAA and BA concentrations on callus induction in *Caladium bicolor*

PGRs (mg L^{-1})		Percentage of callus induction
NAA	BA	
0.25	0.25	33.07 ± 0.27^{g}
0.25	0.50	55.17 ± 0.22^{e}
0.25	1.0	75.60 ± 0.44^{d}
0.50	0.25	81.83 ± 0.22^{c}
0.50	0.50	95.50 ± 1.34^{a}
0.50	1.0	86.97 ± 0.36^{b}
1.0	0.25	77.37 ± 0.36^{d}
1.0	0.5	58.27 ± 0.32^{e}
1.0	1.0	44.53 ± 0.39^{f}
LSD at 5%	-	1.300

Values are mean±standard error of 3 replicates. The data were scored after 4 weeks of culture. Within each column, values followed by the same letters are not significantly different at p=0.05 level according to LSD test

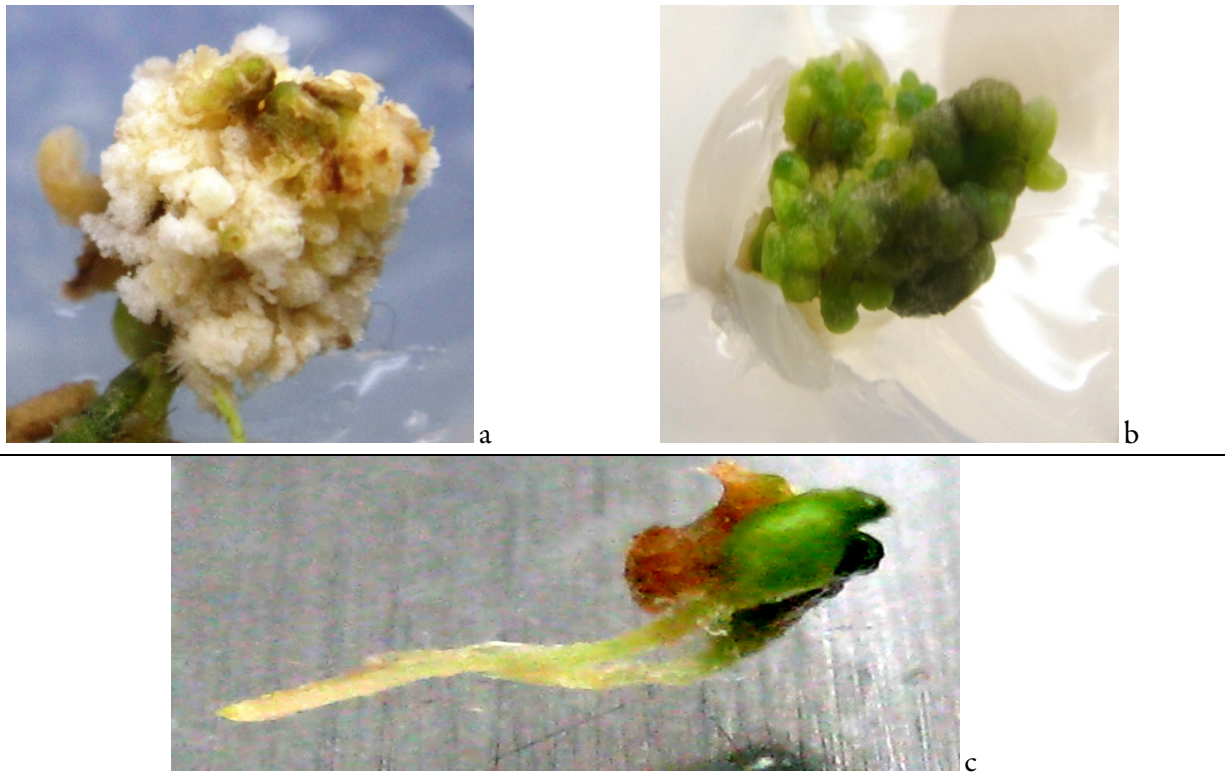

Fig. 1. (a) Tuber-callus induced on NAA (0.5 mg L^{-1}) + BAP (0.5 mg L^{-1}) added medium; (b) embryogenic callus with somatic embryos (arrows); (c) isolated somatic embryos with shoot and root ends (Bar 1a=3cm, 1b=4cm and 1c=2cm)

callus. The embryos (Fig. 1b) started to appear within eight weeks of incubation. Morphologically, the embryos were bipolar 'miniature mango' like structure with green cotyledonary - and root axis (Fig. 1c). The cotyledonary end shows pointed apex with swollen base. In 1.0 mg L^{-1}NAA + 1.0 mg L^{-1} BA added medium the highest numbers of embryos (9.5) were noted (Table 2), although other NAA-BA treatments were also efficient in producing embryos. The embryos later germinated into plantlets, germination percentage being maximum in 1.0 mg L^{-1}NAA + 1.0 mg L^{-1} BA amended medium. Thus, in this present study, a variety of NAA and BAP combinations were noted to be very efficient in inducing callus, embryo and plant regeneration. Mujib *et al.* (1996; 2008) earlier reported that the addition of BA alone or in combination with NAA was very effective for shoot, somatic embryo formation and subsequent growth processes in *C. bicolor*. Ahmed *et al.* (2004) also observed similar high shoot induction response in BA and NAA amended MS medium.

Table 2. Effect of different concentrations of NAA and BA on formation and germination of somatic embryos

PGRs (mg L^{-1})		Number of somatic embryos/callus mass	**Germination percentage
NAA	BA		
0.5	0.5	7.87±0.67^{b}	73.03±0.23^{b}
1.0	1.0	9.50±0.42^{a}	97.60±0.14^{a}
1.5	1.5	7.93±0.53^{b}	67.40±0.32^{c}
2.0	2.0	6.17±0.45^{c}	54.53±0.43^{d}
LSD at 5%	-	0.477	0.787

Values are mean ± standard error of 3 replicates. The number of somatic embryos* and germination percentage** data were scored after 4 and 8 weeks, respectively. Within each column, values followed by the same letters are not significantly different at p=0.05 level according to LSD test

Optimization of alginate and calcium chloride concentration for bead formation and plantlet recovery

Various alginate and calcium chloride concentrations were used to analyze their effect on bead formation and later on plantlet development. A combination of 3.0% sodium alginate and 100 mM calcium chloride proved to be optimum for uniform bead formation (Fig. 2a). In the same sodium alginate and calcium chloride mixture, highest plantlet recovery (92.53%) was noted, followed by the treatment with 3.0% sodium alginate and 125 mM calcium chloride (Table 3). Here, in this current investigation, the polymerizing ability of sodium alginate varied markedly (2.0-3.5%) when used at different concentrations in encapsulating tissues. Block

Table 3. Optimization of sodium alginate and $CaCl_2.2H_20$ (mM) concentrations on conversion frequency of synthetic seeds. The MS medium was added with 0.50 mg L^{-1} BA

Sodium alginate (%)	$CaCl_2.2H_2O$ (mM)	Conversion frequency (%)
2.0	75	41.97±0.58^{i}
2.0	100	46.20±0.39^{h}
2.0	125	49.73±0.04^{g}
2.5	75	51.70±0.19^{f}
2.5	100	59.53±0.15^{e}
2.5	125	67.00±0.55^{d}
3.0	75	71.63±0.60^{c}
3.0	100	92.53±0.75^{a}
3.0	125	82.73±0.33^{b}
3.5	75	70.83±0.29^{c}
3.5	100	50.73±0.11^{fg}
LSD at 5%		1.20

Values are mean ± standard error of 3 replicates with 6 embryos in each replicate. Within each column, values followed by the same letters are not significantly different at *p*=0.05 level according to LSD test

(2003) reported that the polymer concentration, the degree of alginate viscosity, calcium chloride concentration and culturing time are important considerations in determining the permeability, resistance and hardness of the prepared beads and in ultimate success of encapsulation process. In most investigated cases, firm, clear, isodiametric beads of uniform size and shape were achieved using 3.0% sodium alginate and 100 mM calcium chloride. In *C. bicolor,* sodium alginate concentrations below 3.0% produced soft beads, which were difficult to handle, while high concentration (3.5%) produced hard beads, hindered shoot emergence from encapsulated embryos. In several other earlier reports the use of different substances like agar or gel-rite were noted in encapsulating somatic embryos, which improved synthetic seed germination into plants (Maruyama *et al.,* 2003; Utomo *et al.,* 2008). In our experiment, the exposure time to calcium chloride was optimized to be at 15 min (data not shown). High concentration or over exposure of embryos to the calcium chloride facilitated more $CaCl_2$ absorption to the embryo, which caused reduced germination of synthetic seeds and poor growth in field trial (Malabadi and Van Staden, 2005). The protective matrix composition permitted growth of the encapsulated embryo by providing available energy and mechanical resistance to the embryos, while excessively hard matrix caused energy loss with weak or lack of growth (Gonzalez *et al.,* 2004). Brischia *et al.* (2002) reported synthetic seeds' development in M26 apple rootstock; similarly the encapsulation of somatic embryos in producing synthetic seeds was investigated in other horticultural important plants (Choi and Jeong, 2002; Sundararaj *et al.,* 2010). In *C. bicolor*, different combinations of NAA and BA were used in order to optimize the right PGR level on plantlet recovery (Table 4). Among the used combinations, 1.0 mg L^{-1} NAA + 1.0 mg L^{-1} BA showed maximum development of plants (81.13, 89.37 and 96.97% after 6, 9 and 12 weeks of incubation, respectively), followed by 0.50 mg L^{-1} NAA + 0.50 mg L^{-1} BA, while minimum plant development incidence was observed in 0.12 mg L^{-1} NAA+0.12 mg L^{-1} BA. Beside somatic embryos, other explants like encapsulated leaf (71.40%) and petiole (67.43%) also responded well (Table 5) in culture and produced callus when plated on medium. In most studied cases, somatic embryos were used in encapsulation process, however, in recent years, adequate efforts have also been made to explore the possibility of encapsulation of non-embryogenic tissues, *in vitro* derived vegetative propagules such as axillary buds, shoot tips or nodal segments for synthetic seed production (Lata *et al.,* 2009; Micheli *et al.,* 2007; Rai *et al.,* 2009; Singh *et al.,* 2010; Verma *et al.,* 2010). In *Adhatoda vasica* (vasaka), shoot buds were encapsulated in Na-alginate complexing agent (Anand and Bansal, 2002). In our experiment with *C. bicolor,* various explants were encased and upon transfer on MS, the embryo developed into plantlets (Figs. 2b and 2c) and the embryogenic callus proliferated normally after bursting out the encapsulated gel.

Table 4. Effect of various NAA and BA concentrations on plantlet recovery from synthetic seeds in MS medium

PGR (mg L^{-1})	After 6 weeks	After 9 weeks	After 12 weeks
0.12 NAA+0.12 BA	50.93 ± 0.29^{e}	52.73 ± 0.29^{e}	62.13 ± 0.61^{e}
0.25 NAA+0.25 BA	55.77 ± 0.11^{d}	61.43 ± 0.55^{d}	68.77 ± 0.64^{d}
0.50 NAA+0.50 BA	72.20 ± 0.58^{b}	78.33 ± 0.63^{b}	84.67 ± 0.71^{b}
1.0 NAA + 1.0 BA	81.13 ± 0.47^{a}	89.37 ± 0.54^{a}	96.97 ± 0.43^{a}
1.5 NAA +1.5 BA	69.30 ± 0.25^{c}	72.17 ± 0.60^{c}	81.47 ± 0.52^{c}
LSD at 5%	0.83	1.47	1.75

Values are means±standard error of 3 replicates with 6 synthetic seeds in each replicate. Encapsulated condition used: 3.0% sodium alginate + 100 mM $CaCl_2.2H_2O$ + 15 min exposure time. Within each column, values followed by the same letters are not significantly different at p=0.05 level according to LSD test

Table 5. Different explants encapsulation and their callus induction response frequency (%) on MS, amended with 1.0 mg L-1 NAA + 1.0 mg L-1 BA

Explant	Response frequency (%)		
	6 weeks	9 weeks	12 weeks
Petiole	45.60 ± 0.21^{b}	54.93 ± 0.54^{b}	67.43 ± 0.51^{b}
Leaf	52.67 ± 0.23^{a}	62.30 ± 0.32^{a}	71.40 ± 0.19^{a}
Root	24.47 ± 0.74^{c}	27.93 ± 0.60^{c}	32.07 ± 1.17^{c}
LSD at 5%	1.34	1.40	2.16

Values are mean ± standard error of 3 replicates with 6 explants in each replicate. Encapsulated condition used: 3.0% sodium alginate + 100 mM CaCl2.2H20 + 15 min exposure time. Within each column, values followed by the same letters are not significantly different at p=0.05 level according to LSD test

Optimization of storage temperature and duration of synthetic seeds and plantlet conversion

The prepared synthetic seeds were kept at 0 and 4 °C as to optimize the proper temperature for storage (Table 6). Incubation at 4 °C was much more effective than refrigeration at 0 °C, where germination ability was reduced. The synthetic seeds kept at 4 °C for 6 weeks showed good germination (89.40%) in developing plants; the germination frequency was however, declined with longer incubation time i.e. 80.47% and 76.57% were noticed after 9 and 12 weeks respectively. Plantlet recovery was even low (69.53%) when the encased explants were kept at lower temperature of 0 °C, the recovery percentage was further down with time i.e. 49% recovery was noted after 12 weeks of storage at 0 °C. In *Pinus patula,* satisfactory conversion (61-73%) was noted from encapsulated embryos, kept at 2-4 °C for four months, while storage at higher temperatures (e.g. 27 °C) reduced plantlets conversion to 6% (Malabadi and Van Staden, 2005); the observation is very similar to our present findings. The non-encapsulated embryos did not germinate at all after storage at 0 °C and 4 °C, very similar to observation made by Katouzi *et al.* (2011). In a recent analysis, Mehpara *et al.* (2012) observed maximum plantlet development in *C. roseus* when synthetic seeds were kept at 4 °C. Bernard *et al.* (2002) and Kaviani (2010) reported that high level of sucrose addition in alginate matrix is necessary for cryogenic storage (encapsulation-dehydration) as sucrose increases tolerance to dehydration and improves tissue viability during embryo germination process. Here, the *Caladium* plantlets (Fig. 2d) obtained from synthetic seeds grew well in MS and are very similar to the mother plant. This is the first ever report of synthetic seed production by encapsulation and the study also described *in vitro* short-term storage of synthetic seeds and plantlets development in *C. bicolor*.

Table 6. Effect of different storage temperatures on plantlet regeneration from synthetic seeds. MS medium was fortified with optimized 1.0 mg L^{-1} NAA + 1.0 mg L^{-1} BA

Temperature (°C)	After 6 weeks	After 9 weeks	After 12 weeks
4	89.40 ± 0.35^{a}	80.47± 0.25^{a}	76.57 ± 0.45^{a}
0	69.53 ± 0.39^{b}	56.17± 0.50^{b}	49.00 ± 0.25^{b}
LSD at 5%	0.76	1.11	1.63

Values are means±standard error of 3 replicates with 6 synthetic seeds in each replicate. Encapsulated condition used: 3.0% sodium alginate + 100 mM $CaCl_2.2H_2O$ + 15 min exposure time. Within each column, values followed by the same letters are not significantly different at p=0.05 level according to LSD test

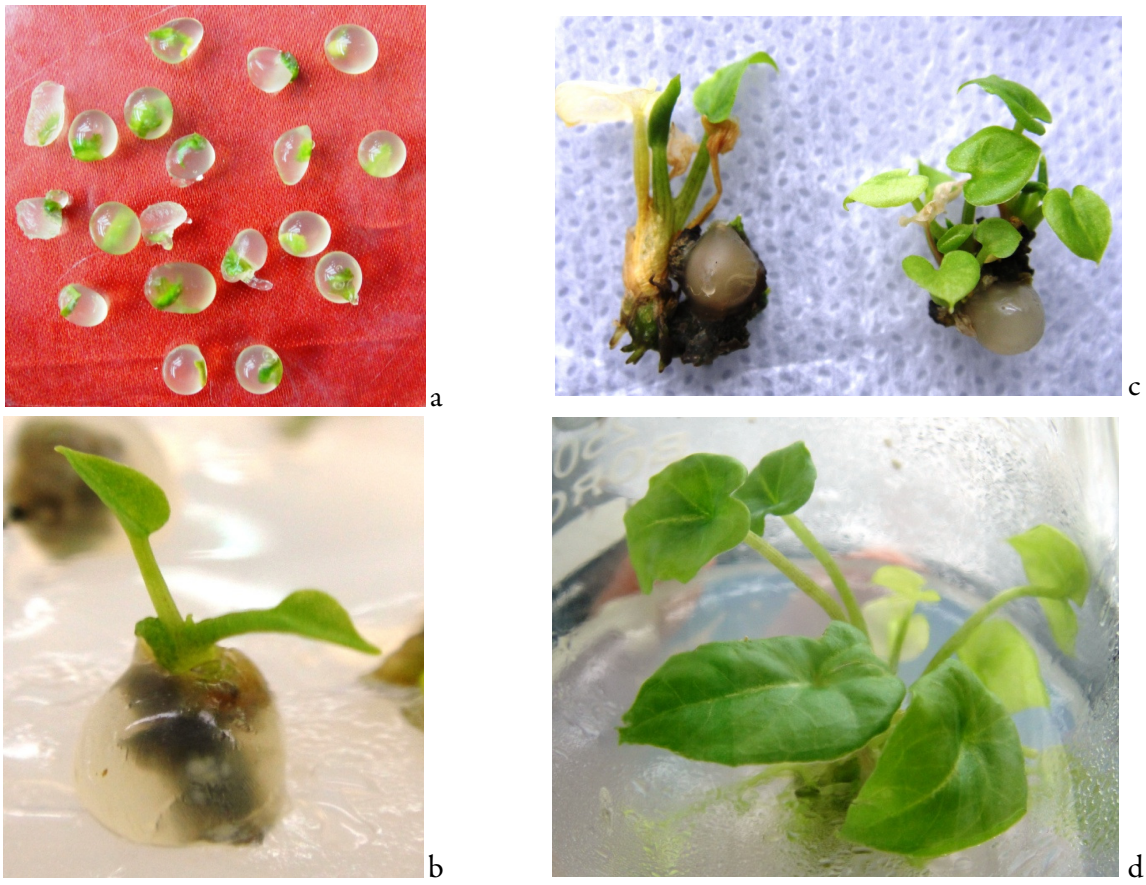

Fig. 2. (a) Encapsulated beads containing various explants; (b) & (c) plantlets coming out from encapsulating matrix; (d) regenerated plantlet from synthetic seeds (Bar 2a=2mm, 2b=1cm, 2c=2cm, 2d=2cm)

Conclusions

In conclusion, the present study established an effective technique for synthetic seed production and short-term storage in *C. bicolor*. The optimized methodology may provide an efficient way in preserving important elite *C. bicolor* germplasm for short to medium-term basis without any loss or alteration of morphological traits. The precise addition of cryo-protectants may increase the storage time further. On requirement, the *Caladium* may be produced in masses and the important germplasm can be transferred and exchanged between laboratories globally. The long-term storage of encapsulated explants at low temperature and in minimal nutrient media could be an effective approach in rejuvenating floriculture industry as well.

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