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RESEARCH ARTICLE

In vitro morpho-regeneration, mass multiplication and bulb production in calla lily (*Zantedeschia* spp.) cultivars

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Abstract

This study explored *in vitro* morpho-regeneration responses and mass multiplication through long-term subculture in calla lily (*Zantedeschia* spp.) cv. Celesta, Gold Affair and Majestic Red. Significantly, the highest (91.40 %) and lowest (80.02 %) clean cultures were recorded in cv. Majestic Red and Gold Affair, respectively. In morphogenetic responses, the highest shoot numbers of 6.18, 4.45 and 5.24 (on 0.1 mg/L 2,4-D and 1.5 mg/L BAP) and the highest shoot lengths of 4.36, 3.99 and 3.87 were recorded in cv. Celesta, Gold Affair and Majestic Red, respectively, after a short span of 13–15 days of incubation. There was a reduction in the average number of shoots and shoot length in all cultivars during long-term subculture. All cultivars showed 100 % in vitro root induction. In hardening, survival varied with cultivars; the maximum (84.81 % and 66.54 %, respectively) survival in primary hardening and shade net was observed in cv. Majestic Red. The hardened plants were used for bulb production under a polyhouse. The highest 'A' grade bulbs were recovered from cv. Gold Affair (32.59 %). The 'A' and 'B' grade tubers of all cultivars have shown good flowering ability and the 'C' grade bulbs were weak in setting flowers. Overall, the study demonstrates efficient *in vitro* morpho-regeneration and sustained responses during long-term subculture for mass multiplication and bulb production in calla lily. This study presents a micropropagation system, which is simple, low-cost, viable and sustainable for large-scale production of healthy and quality calla lily plantlets as well as bulbs with flowering ability.

Keywords: bulbous plants; calla lily; gold affair; hardening; *in vitro*; majestic red; micro propagation; morpho-responses; PGRs

Introduction

Calla lily (*Zantedeschia sp.*; family Araceae) is an herbaceous flowering plant native to South Africa. It is cultivated for marketing as cut flowers and potted plants due to its attractive spathe. Warmer temperatures and high light intensities enable growers to produce their flowers all year round in a relatively short production cycle. Further, landscapers extensively use this flower for improving aesthetic value. Hence, there is a great demand for quality planting material. It is conventionally propagated by tubers, resulting in a limited multiplication rate and leading to limited availability of planting material (1). Conventional propagation is further limited by fungal, bacterial and viral infections during plant multiplication. These infections can reduce floral and rhizome yield and in severe cases, may cause plant death (2). Therefore, plant tissue culture is the only technique that allows fast clonal propagation and provides healthy and uniform plants (3, 4). Tissue culture-derived microtubers provide the highest level of propagule sanitation. They are directly plantable and suitable for plug systems adapted to automation, while minimising mechanical injury and reducing soft rot-like disease incidence (5). Though the system of micropropagation improves multiplication, broader application has been reduced by its enhanced cost of production, which is primarily due to low growth of plantlet, a high percentage of culture media contamination and laborious work (6, 7). Also, *in vitro* propagation of Calla lily cultivars has not been easy due to contamination of the explants (8) and hence proper standardisation of the protocol for culture establishment and multiplication is necessary. Several studies on *in vitro* micro propagation of calla lily (3, 9–16) have focused mainly, concentrating mainly on clean culture establishment and assessment of plant growth regulators (PGRs) for shoot and root regeneration. Importantly, different genotypes of calla lily have different responses to PGRs and problems like contamination in initiated cultures and low shoot multiplication rate still exist (17). Further, little information is available on hardening and no information is available on morpho-regeneration responses through long-term subcultures, which are required for large-scale production of planting material for commercial sale. Therefore, the present study was planned to explore *in vitro* morpho-regeneration responses on various PGR combinations and long-term subculture in cultivars of Calla lily employing bulbs from field-grown plants and bulb production from hardened plants.

Materials and Methods

Media preparation and sterilisation

The culture medium was prepared using MS salts, organics (18), sucrose 30 g/L, adjusted pH to 5.8 ± 0.1 and solidified with 6.5 g/L agar. The culture initiation medium (CIM) was prepared by adding

2,4-D 0.2 mg/L and BAP 1 mg/L; the shoot multiplication and shoot elongation medium (SMSEM) was prepared by adding 2,4-D 0.1 mg/L and BAP 1.25 mg/L; and the rooting medium (RIM) was prepared by adding BAP 1.5 mg/L and IAA 0.3 mg/L. The 30–35 mL of medium was dispensed into pre-sterilised 250 mL glass culture bottles with caps, autoclaved in a steam steriliser (Nat Steel Pvt. Ltd., India) at 121 °C and 15 lbs for 18 min and stored in a media storage room at ambient temperature for 7–10 days before usage. All the chemicals used in the study were procured from Hi-Media Laboratory Chemicals, India.

Establishment of a clean culture

'A' (45 cm diameter) grade sprouted bulbs of cv. Celesta, Gold Affair and Majestic Red were selected from cold storage (7–8 °C) and washed two times in sterile water for 15 mins. Later, immersed in Bavistin (200 mg/100 mL of water) and citrimide (50 mg/100 mL) with two drops of Tween-20 and kept in a shaker for 1 hr. Then, bulbs were washed 3–4 times with sterile water. Then bulbs were soaked in 100 ppm GA_3 for 2 hr; later the outer layer was peeled off with a scalpel blade, immersed in sodium hypochlorite (2 %) for 5 mins, then washed two times with sterile water at 2 min intervals and taken into a Laminar Air Flow unit (Servo Scientifics, Bengaluru) placed in a culture initiation room. Bulbs were taken into a new sterile flask, dipped in 70 % ethanol for 30 sec and washed two times with sterile water at 2 min intervals. Then immersed in sodium hypochlorite (2 %) for 5 min and washed two times in sterile water at 2 min intervals. They were trimmed on all sides of the bulb to bring it to a size of 1.5–2 cm in diameter. Then, inoculated on CIM, closed the cap tightly, sealed with cling film and incubated in light (12 hr of 40 W tube lights) at 25 ± 1 °C. This experiment was carried out in a CRD design with three replications. Observations on clean cultures, contamination and sprouting in initiated cultures were recorded at two-week intervals up to 6 weeks.

Assessment of PGRs for morpho-regeneration in calla cultivars

Cultures of all three cultivars showing multiple shoot sprouts were divided into clumps (0.5 cm^2) used as explants. Six clumps were inoculated per bottle containing the above-mentioned basal culture medium along with different levels and combinations of 2,4-D (0.1, 0.2 and 0.5 mg/L) and BAP (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L). This experiment was carried out in a three-factorial CRD design with three replications. Observations on initiation response, shoot numbers and length of shoot were recorded during subculture for the next cycle (13–15 days).

Mass multiplication through long-term subculture

Cultures of all three cultivars showing multiple shoot sprouts were divided into clumps (0.5 cm^2) used as explants. Six clumps were inoculated per bottle containing the above-mentioned basal culture medium along with 0.1 mg/L 2,4-D and 1.5 mg/L BAP (the best treatment in the above experiment) at 13-15 day intervals for 10 passages. Observations on initiation response, shoot numbers and length of shoot were recorded during subculture for the next cycle.

Rooting and hardening

Grown-up shoots (more than 3 cm in height) were singled out, cleaned at the base and ten shoots were inoculated into a bottle of RIM. Contamination-free rooted plantlets in bottles were sorted out and sent for hardening. Plantlets were taken out, washed carefully and thoroughly to remove agar residues so as not to damage the shoot/root, planted in trays containing soil rite and coir pith mixture (1:1) and kept in a polyhouse maintained at 25-26 °C and 85 % relative humidity for 22–25 days (for primary hardening). They were watered at 3–4 day intervals. Primary hardened plants were transferred into polythene covers containing soil, sand and FYM (1:1:1) and kept under a shade house (50 % for secondary hardening). They were watered once every 2–3 days. This experiment was carried out in a CRD design with three replications. Observations on root induction were recorded after eight days of inoculation. Plants survived in the greenhouse (primary hardening) and the shade house was recorded after four weeks.

Recovery of bulbs and assessment for flowering ability

After 80–90 days of transplanting, once shoots started drying, bulbs were separated from roots by gently disturbing the soil in the cover, bulbs were sorted out into A (4 cm diameter and more), B (3–4 cm diameter) and C (<3 cm diameter) grades based on size, treated with 100 ppm GA_3 and sown in soil mixture beds for assessment of flowering ability. This experiment was carried out in a three-factorial CRD design with three replications. Observations on flowering ability were recorded after 45 days of bulb sowing in nursery beds and on the grade of bulbs during recovery of bulbs.

Data collection and analysis

All experiments were repeated two times for the authentication of results and data were subjected to ANOVA for statistical analysis (OP Stat website, http://opstat.somee.com/opstat/). The results are presented as averages, percentages and numbers as necessary, along with significance at <0.01 %

Results and Discussion

Clean culture establishment

Plantlets produced from tissue culture are an alternative for tuber production in Calla lilies and hence production of plantlets *in vitro* has become an important activity. However, in crops belonging to the Araceae family, especially in tubers of *Zantedeschia* spp., there is a huge problem in the culture initiation as appearance of contamination at the initial stage, which is mainly due to endogenous pathogens (9, 19). In this study, the sterilisation protocol standardised in our laboratory (unpublished data) has shown a significant effect on the establishment of clean cultures in different cultivars. The highest and lowest percentages of clean cultures were 91.40 and 80.02, respectively, recorded in cv. Majestic Red and Gold Affair, respectively (Table 1). Bulbs started bulging in 12, 21 and 16 days after inoculation and shoots started appearing after 28, 45 and 36 days in cv. Celesta, Gold Affair and Majestic Red, respectively. The bacterial and fungal contamination in initiated cultures has varied with cultivar. However, the contamination was efficiently controlled by the surface sterilisation procedure employed (Fig. 1A). The earlier studies showed that the sterilisation of explants taken from the tuber of *Zantedeschia* spp. using sodium hypochlorite (10 % solution) resulted in 20 % of 'clean' explants free from contamination (20). Immersion for a period of 15 min reduced contamination of explants (27 %) (3). In this study, the timings and concentration of sterilising agent, explants preparation and water washes were found to be optimum and hence it was a better protocol for the establishment of clean cultures than earlier reported studies.

Table 1. *In vitro* response of calla lily cultivars in the establishment of clean cultures

Cultivars	No. of bulbs used	Initiation of response (days)	Clean cultures	Contamination		Shoot vigour	Initiation shoots (Days)
				Bacteria	Fungus		
Celesta	25	12	84.60	13.00	3.2	++++	28
Gold affair	25	21	80.20	20.40	0.6	++	45
Majestic Red	25	16	91.40	10.20	0.6	+++	36
SE (m)	-		1.44	2.81	1.74		
C.D	-		0.469**	0.912**	0.565**		

**Highly significant

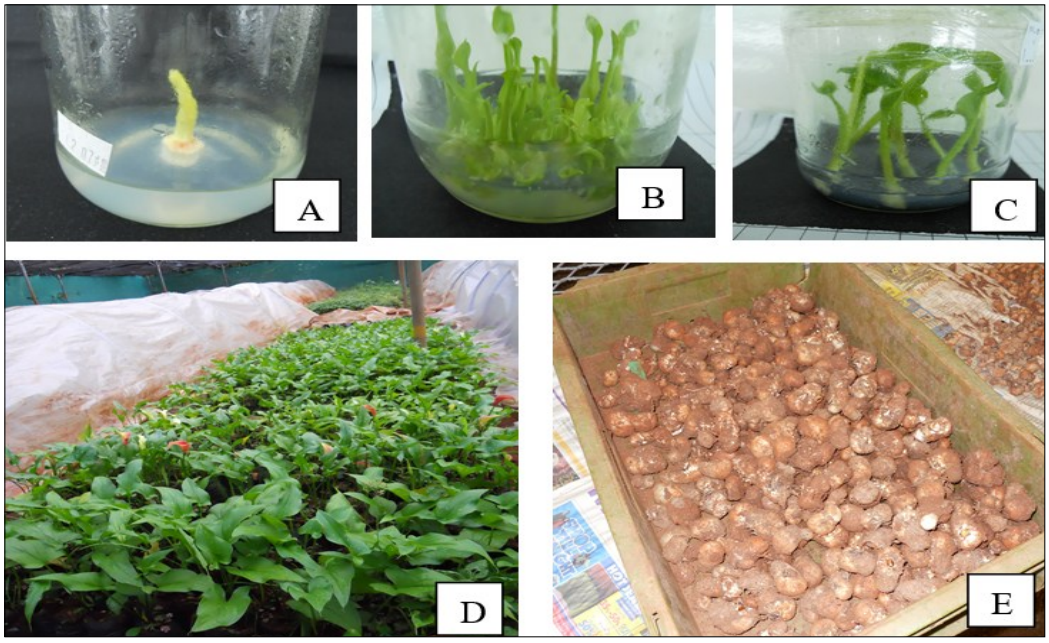

Fig. 1. A. Initiation of culture using bulb; B. Multiplication of shoot culture; C. *In vitro* rooting in shoots; D. Hardened plants; E. Harvested bulbs.

Assessment of PGRs for morpho-regeneration in calla lily cultivars

Several reports have suggested that the multiplication rates through micropropagation can be regulated by varying the type and/or concentration of plant growth hormones in the culture medium (21). However, in tissue culture, the conditions which help the formation of an adventitious bud (increased plant multiplication) would normally reduce plant growth. Hence, balanced multiplication rate along with good plant growth is very important for developing an efficient *in vitro* protocol for large-scale multiplication of mother tubers (22). In this study, there were significant differences in the number of shoots and shoot length among cultivars affected by PGR combinations. Initially, the appearance of tiny shoots was observed, which was an indication of shoot initiation in response to the medium. The lowest days taken for initiation of response were 2.5, 4.5 and 4.5 days (on 0.2 mg/L, 2, 4, D and 1.5 mg/L BA) in cv. Celesta, Gold Affair and Majestic Red, respectively. Later, multiple shoots started growing from the base of the explants (Fig. 1B). The highest shoot numbers of 6.18, 4.45 and 5.24 and the highest shoot lengths of 4.36 cm, 3.99 cm and 3.87 cm were recorded in cv. Celesta, Gold Affair and Majestic Red, respectively, after 13–15 days of incubation. Further, callusing was observed with an increase in the level of 2,4-D as well as BAP (Table 2).

Similarly, the effectiveness of BAP alone (23, 24) and/or in combination with auxins (IAA, NAA and 2, 4-D) (13, 16, 25) for the multiplication of plants of the family Araceae was confirmed by many earlier studies. An average of 3.8 and 3.2 shoots per explant with BA (8.87 µM-1.5 mg/L) or TDZ (4.54 µM), respectively, was recorded in *Zantedeschia albomaculat* (26). But they found that increasing concentrations of cytokinins often led to lower proliferation rates and stunted shoot growth. 3.33 shoots with 3.77 cm shoot length in *Zantedeschia rehmannii in vitro* cultures after six weeks of incubation have been reported (10). Also, 2.6 shoots with as high as 22.19 µM BAP have been reported in Calla lily *(Zantedeschia aethiopica* L.) (19). The highest multiplication of shoots in cultivar Orania and Sunclub (15.33 and 4.32 average number of shoots, respectively) was noticed on culture media containing BAP (2.5 mg/L) and Kinetin (1.5 mg/L). The higher length of shoots in cultivar Orania and Sunclub (94.00 mm and 95.20 mm average length of shoots, respectively) was obtained in culture medium containing 3 and 1.5 mg/L BAP and Kinetin, respectively, with incubation of 30 days (14). The addition of a low concentration of auxins, 1 mg/L each of NAA and IBA, produced an average number of 14.7 shoots per explant with an average length of 4.1 cm after 40 days of subculture (11). Hence, it is confirmed that multiplication rates through micro propagation were regulated by varying the type and/or concentration of plant growth hormones in the culture medium (21, 22-27). Similarly, this study recorded high, efficient and consistent shoot multiplication (both number of shoots and shoot length) on MS medium containing auxin (2,4-D at 0.2 g/L) and cytokinin (BAP at 1.5 mg/L) in all cultivars. It may be because of the best and balanced hormonal combination having very low concentration of auxin (2,4,D- replacement for NAA/IBA) along with medium concentration of cytokinin (BAP), which suits for increased shoot multiplication.

Mass multiplication through long-term subculture

The protocol standardised produced vigorous and high shoot numbers and lengthy shoots in the first subculture. The vigour as well as the average of these responses in the next nine passages was less in all cultivars (Fig. 2). However, the reduction in multiplication rate and vigour was quite marginal, which did not affect the quality of the plants. In the first (7.4 and 5.95 cm) and

Table 2. *In vitro* mopho-response of calla lily cultivars on different growth regulator combinations

mg/L		Initiation response			No. of Shoots			Shoot Length			Callus		
2, 4-D	BAP	C	GA	MR	C	GA	MR	C	GA	MR	C	GA	MR
0.1	0.5	8.50	7.50	7.50	1.04	0.73	0.96	0.64	0.56	0.62	-	-	-
	1	6.50	7.50	7.50	3.07	2.78	2.92	1.98	1.78	1.86	-	-	-
	1.5	5.50	7.50	7.50	6.18	4.45	5.24	3.88	3.55	3.29	-	-	-
	2	6.50	8.50	7.50	5.05	3.97	4.76	3.84	3.44	3.18	-	-	-
	2.5	8.50	10.50	9.50	2.16	1.93	2.08	2.97	2.35	2.26	-	-	-
0.2	0.5	5.50	5.50	5.50	0.67	0.44	0.56	1.06	0.81	0.87	-	-	-
	1	5.50	5.50	5.50	0.98	0.89	0.95	2.97	2.92	3.15	-	-	-
	1.5	2.50	4.50	4.50	1.96	1.65	1.74	4.36	3.99	3.87	-	-	-
	2	4.50	3.50	3.50	1.90	1.37	1.45	2.00	1.88	1.67	++	++	++
	2.5	6.50	8.50	7.50	0.34	0.22	0.25	2.16	1.71	1.90	++	+++	++
0.5	0.5	11.50	12.50	11.50	0.55	0.44	0.49	0.30	0.24	0.26	++++	++++	++++
	1	11.50	12.50	11.50	0.85	0.77	0.79	0.40	0.44	0.47	++++	+++++	++++
	1.5	9.50	11.50	9.50	1.26	1.04	1.18	1.12	1.01	0.76	+++	++++	+++
	2	9.50	11.50	9.50	2.47	2.25	2.02	2.28	2.52	2.39	++	+++	++
	2.5	6.50	7.50	6.50	3.54	2.94	3.26	3.02	2.70	2.58	+	++	+
Factors		C.D.	SE(m)		C.D.	SE(m)		C.D.	SE(m)				
2, 4-D		0.21	0.08		0.07	0.03		0.07	0.03				
BAP		0.27	0.10		0.10	0.03		0.09	0.03				
2, 4-D × BAP		0.47	0.17		0.16	0.06		0.16	0.06				
Cultivars		0.21	0.08		0.07	0.03		0.07	0.03				
2, 4-D × Cultivars		0.36	0.13		0.13	0.05		0.12	0.04				
BAP × Cultivars		0.47	0.17		0.16	0.06		0.16	0.06				
2, 4-D × BAP × Cultivars		0.81	0.29		0.28	0.10		N/A	0.10				

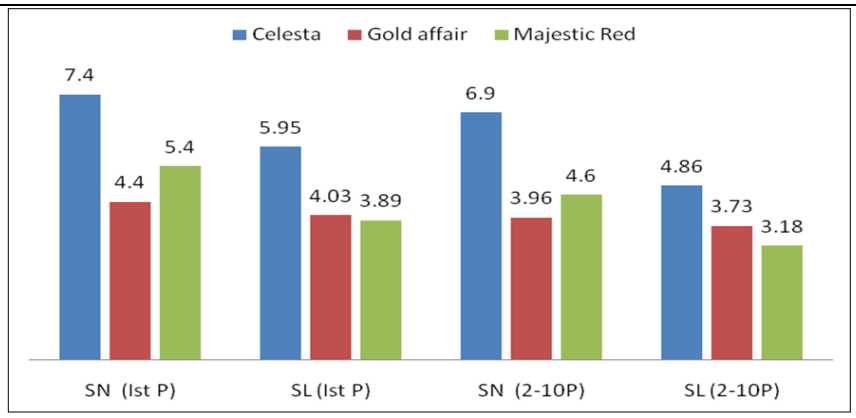

Fig. 2. *In vitro* response in long-term subculture in calla lily cultivars. *Index: SN-IstP: Shoot numbers per explant in first passage; SL-IstP: Average shoot length per explant in first passage; SN (2-10P): Average shoot length in 2nd to 10th passage; SL(2-10P): Average shoot length in 2nd to 10th passage.

subsequent nine subcultures (6.9 and 4.86 cm), the highest shoot numbers and shoot length per explant were recorded in cv. Celesta, respectively. A multiplication rate of 3.0 on culture media containing BAP (22.2 and 44.4 µM) or kinetin (11.6 and 23.2 µM) and 4.0 on media containing 2iP (12.3-49.2 µM) in was recorded in 'spreng' and other cultivars were recorded (26–28). The present study stands very unique to date in recording morpho-regeneration response in long-term *in vitro* subculture, which has not been reported earlier. The multiplication rates in long-term subcultures were also good (2.8–3.5 times, data not presented). Nevertheless, results are encouraging and play a very important role in the large-scale multiplication of planting material for commercial sale. To the best of our knowledge, long-term morpho-regeneration responses in calla lily have not been previously reported.

Rooting and hardening

Root induction was observed in 100 % of plantlets (Fig. 1C). Most of the earlier studies reported similar results on *in vitro* root induction (3, 11, 28). In this study, significant differences were recorded in the survival of plantlets both in primary and shade net (secondary) hardening among cultivars. A maximum of 84.81 % and 66.54 % plants survived in the primary and shade net in the cv. Majestic Red (Table 3). The hardened plants were ready for sale

Table 3. Bulb recovery from *in vitro* regenerated plantlets and the flowering ability of bulbs

Cultivar	Rooting (%)	Hardened plants (%)		Bulbs recovery (%)			Flowering in bulbs (%)		
		Primary	Shade net	A	B	C	A	B	C
Celesta	100	90.55	69.78	24.81	35.43	39.85	100.00	89.13	35.43
Gold affair	100	82.18	64.24	32.59	33.39	34.02	100.00	97.26	38.37
Majestic Red	100	79.81	61.54	29.35	36.47	34.18	100.00	100.00	47.91
S.Em	-	2.290	1.766	S.Em (cultivars)	0.714			0.478	
CD	-	7.056*	5.442*	CD (cultivars)	2.08**			1.39**	
				S.Em (Bulbs)	0.714			0.478	
				CD (Bulbs)	2.08**			1.39**	
				S.Em (Cultivar × Bulbs)	1.23			0.82	
				CD (Cultivar × Bulbs)	3.60**			2.41**	

**Highly significant; *Significant

after 45–50 days of bringing them out of the laboratory (Fig. 1D). The survival of plantlets in primary hardening was good due to optimum temperature and humidity maintained continuously. Only the light conditions are regulated by the shade net and hence, the survival rate might have been reduced. Similarly, the survivability of the majority of micropropagated plants is poor in a greenhouse during the transplanting from *in vitro* conditions because of their sensitivity (14). Further, hardening losses in shade is more because of problems such as lower relative humidity, higher light intensity and infections in *in vitro* produced plantlets compared to laboratory conditions. However, the present study was successful in hardening *in vitro* plants efficiently.

Bulb recovery and assessment of flowering ability

In this study, there was a significant difference in the recovery of A, B and C grade tubers (Table 3; Fig. 1E). The highest 'A' grade tuber recovery was recorded in cv. Gold Affair (32.59 %) and the lowest of it was recorded in cv. Celesta (24.81 %). Similarly, earlier 25 % and 52 % plants in pots and soil beds produced more than 3 cm tubers in diameter, respectively (22). Similarly, the flowering in calla lily was dependent on the size of the bulb and it was improved with GA3 treatment (22, 29, 30). In the present study, 100 % of plants derived from GA_3-treated 'A'-grade tubers flowered across all cultivars (Table 3). Additionally, B and C grade tubers of cv. Majestic Red showed 100 % flowering, whereas in the other two cultivars, flowering ability was reduced, which could be due to genotypic differences in response to flowering. Usually, calla lily growers prefer a high proportion of large-sized tubers because of their flowering ability. Generally, plantlets produced through in vitro culture require two ex vitro growth cycles to attain flowering (1). Further, they have also mentioned that in vitro produced plantlets may take two seasons to reach flowering, in the first cycle produced tiny tubers and in the second cycle produced tubers with flowering ability. However, the occurrence of flowering from tubers derived from in vitro plants during the first growth cycle in the present study corroborates earlier findings (22).

Conclusion

In conclusion, the present study revealed morpho-regeneration responses on various PGRs in different cultivars and in long-term *in vitro* subcultures. The hardened plantlets were successful in producing bulbs with flowering ability. Finally, the protocol presented (Fig. 3) can be effectively used for the large-scale production of high-quality planting material and bulb production in calla lilies. Moreover, the protocol may also be utilised for the in vitro conservation of calla lily germplasm.

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Authors' contributions

DPP carried out research and prepared. SPP provided varietal details and helped in statistical analysis of data. RTP and MK provided support in preparing manuscript. All authors read and approved the final manuscript.

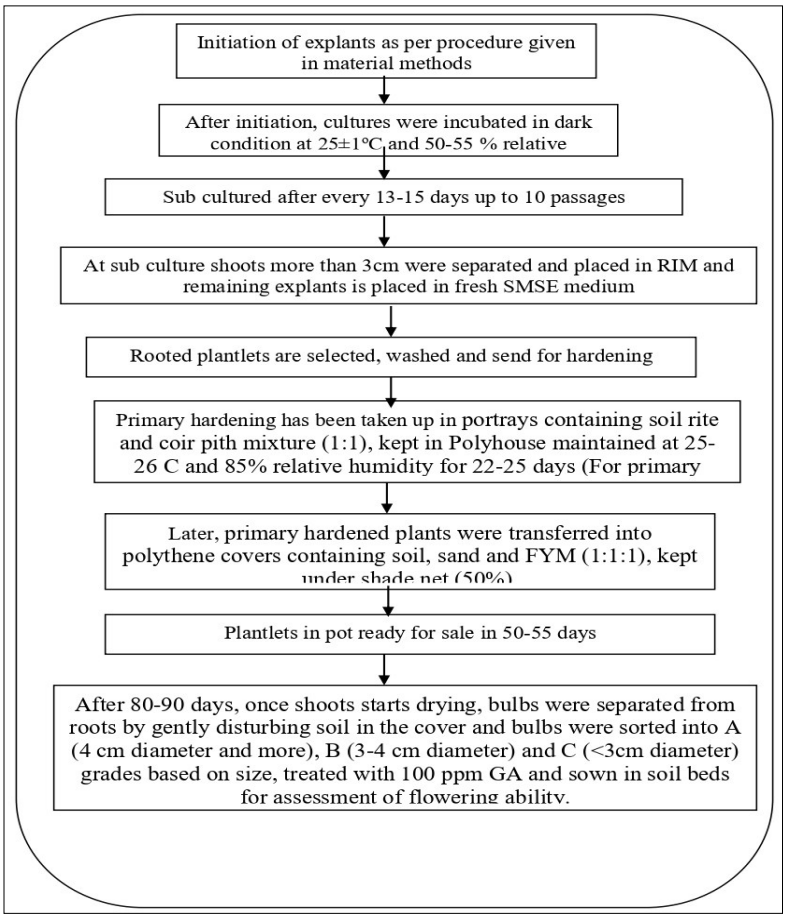

Fig. 3. Protocol for *in vitro* mass multiplication of calla lily.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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