

***In vitro* cormlet production- an efficient means for conservation in *Ensete superbum* (Roxb.) Cheesman**

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Abstract

Ensete superbum from the family Musaceae is commonly known as Kallu vazha (wild/ rock/cliff banana). The species holds a precise position in the field of medicine for its anti-hyperglycemic, anti-diuretic and spermicidal potential as well as ornamental value in botanical gardens. Due to deforestation, habitat fragmentation, indiscriminate harvesting for commercial gain, absence of suckers, and recalcitrant nature of seeds; this species is facing a drastic reduction in its propagation. The present study developed a protocol for the production of cormlets from explants isolated from inflorescence. The explants were cultured on MS media supplemented with 4mg L⁻¹ BAP and 1.5 mg L⁻¹ KIN and an average of six to ten cormlets were produced/ explants within eight weeks. Shoot induction occurred from the cormlets on MS medium with 3mg L⁻¹ IBA and 1.5 mg L⁻¹ BAP. Cormlets inoculated on MS medium supplemented with 1000 mg L⁻¹ glutamine for a period of four weeks enhanced the size of cormlets which in turn increased the number of shoots. An average of ten multiple shoots were obtained on MS medium supplemented with 5 mg L⁻¹ BAP. Maximum rooting was obtained on half strength MS medium with 3 mg L⁻¹ IBA, 0.1 mg L⁻¹ BAP and 1% activated charcoal. The plantlets were transferred to Knop's solution for acclimatization. Rooted plants were hardened successfully in cocopeat along with sand in 1:1 combination and transferred to soil with 98% survival rate.

Key words: *Ensete superbum*, conservation, micropropagation, cormlets, glutamine, Knop's solution.

Introduction

Ensete superbum (Roxb.) Cheesman, a threatened medicinal plant, commonly known as rock, wild, or cliff banana, shows dissimilarities with *Musa* both morphologically as well as genetically even though residing under the same family musaceae. The genus *Ensete* which comprises of nine species having a chromosome number 2n= 18 (Cheesman 1947, Simmonds 1960, 1962). It is a monocarpic, non- stoloniferous perennialshrub usually propagated by seeds. It is endemic to the Western Ghats, the Aravalli range and North-eastern hills of India and Northern Thailand (Vasundharan *et al.*, 2010). *Ensete* possess a swollen base called corm, a pseudo stem, very large broad leaves with prominent midribs, inflorescence that resembles a lotus flower, all contribute to its ornamental value in botanical garden.

Its endosperm is used as remedy for different ailments such as diabetes, kidney stone and leucorrhoea. Presence of non-steroidal phytosterole-4-hydroxyl-3-methyl-hex-5-enyl is reported to lower cholesterol when it is used as a food additive (Vasundharan *et al.*, 2013, Kachroo *et al.*, 2011). The ethanolic seed extract possess appreciable spermicidal potential and can be used as an effective contraceptive (Sarwar *et al.*, 2014). It was reported that aqueous seed extract of *Ensete* can inhibit the growth of calcium hydrogen phosphate dehydrate, thereby preventing urinary stone formation (Diana *et al.*, 2013). The plant or plant product was used as a prescribed medicine by indigenous practitioners for nineteen etiological symptoms (Vasundharan *et al.*, 2015).

Its unripe fruits and flowers were used as vegetables and was reported that its antioxidant activity was found higher than commercial banana after thermal processing (Sasipriya *et al.*,

2014). It is commonly known as 'false banana' since unlike commercial bananas, its pseudo stem and corm are edible rather than ripe fruits and possess high starch compared to potato (Birmeta *et al.*, 2004). Sethiya *et al.* (2016) reported that the methanolic extract of its pseudostem showed highest concentration of phenolics, flavonoids and had significant anti-oxidant activity. TLC fingerprint is also used in characterization of plant extract for standardization (Sethiya *et al.*, 2016). Unregulated harvesting of seeds, seedlings from its natural habitat and nonscientific leaf harvesting drastically retarded flowering and fruiting which in turn reduced its population (Bhise *et al.*, 2015). Hence it is considered as rare, relict, endangered, threatened and conservation concern species (Vasundharan *et al.*, 2011). Vasundharan *et al.* (2015) reported the need of further initiatives from the authorities to empower Good Agriculture and Collection Practices (GACP) for medicinal plant recommended by world health organization and to study the present population status of the species by IUCN's guidelines and recommendations to conserve this Wild Banana species struggling for survival.

A few reports on clonal propagation and somatic embryogenesis were reported for this species (Mathew *et al.*, 1996, 2000, 2002). Somatic embryogenesis from male floral apices of *Ensete* was published by Kulkarni *et al.* (2002). Negash *et al.* (2001) reported the *in-vitro* conservation of *Ensete ventricosum* shoot tips for six months. The present paper reports *in-vitro* production of cormlets, which is the key organ for multiple shoot induction in *Musa* species, as a tool for micropropagation and conservation of this species.

Materials and methods

Plant materials: Inflorescence (apical meristem with male floral buds) having 22 cm length and 30-32cm diameter (Fig.1A), were collected from three to four years old wild plants growing at Sethathodu, Pathanamthitta district, Kerala, India (9.34°N, 76.99°E), during of November 2014. The outer bracts were removed to attain an inflorescence with 6cm length and 3 cm diameter, washed first in 10% labolene (Qualigens, India) for 15 minutes and then in running tap water for 30 minutes. It was surface sterilized by 0.1% HgCl₂ for ten minutes in laminar air flow hood, rinsed with sterilized double distilled water for five

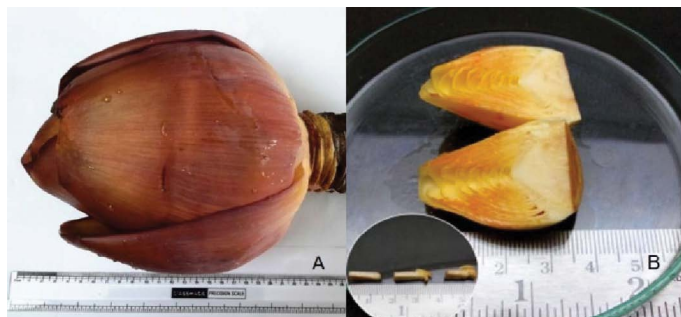


Fig. 1. (A) Inflorescence explant. (B) 3.5 cm represents explant with apical meristem. Fig.1 (B Inset) represents explants of approximately 1cm in size

times, five minutes for each wash. Both ends were trimmed to get a final size of 3.5 cm length and 1.5 cm diameter, and were divided longitudinally into two pieces. Explants of approximately 1cm in size were isolated from the basal region aseptically in such a way that each piece consisted of basal apical meristem (Fig.1B).

The explants were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with 6-benzylaminopurine (BAP) at concentration of 1, 2, 3, 4 or 5 mg L⁻¹ alone or in combination with 1, 6-furfuryl amino purine (KIN) at concentration of 0.5, 1, 1.5 or 2 mg L⁻¹ respectively. The pH of the medium was adjusted to 5.8, solidified with 0.7% agar (wv⁻¹) and sterilized by autoclaving at 121°C at 15 lbs. pressure for 15 minutes. The explants were inoculated horizontally on the medium. Cultures were maintained under 16 hours photoperiod at 25±1°C and 60 - 80% relative humidity. The light intensity of 2500 lux was provided by means of white fluorescent light. The response was observed after four weeks of culturing and number of cormlets per explants were recorded.

Cormlets obtained were sub cultured on MS medium supplemented with IBA at concentration of 1, 2, 3, or 4 mg L⁻¹; along with 1.5 mg L⁻¹ BAP to induce shoot regeneration. The average number of shoot buds, length of longest fleshy leaves, length of longest leaf sheaths and number of normal shoots were recorded at two weeks interval up to 12 weeks. .

The cormlets were inoculated on MS medium supplemented with 3 mg L⁻¹ IBA, 1.5 mg L⁻¹ BAP and 500, 1000 or 1500 mg L⁻¹ glutamine. Average number of shoot buds, length of longest fleshy leaves, length of longest leaf sheaths and number of normal shoots were recorded at two weeks interval up to 12th week. Photographs of free hand sections of the cormlets were taken using image analyzer (Leica DM 2000) to analyze the mode of shoot regeneration.

Regenerated shoots were transferred for shoot multiplication on MS medium supplemented with BAP at concentration of 3, 4, 5 or 6 mg L⁻¹ alone or in combination with IBA (at concentration of 0.5, 1, 1.5 or 2 mg L⁻¹), respectively. Observations were recorded at one week interval for a month.

Shoots, after attaining a height of 4-5 cm were transferred to rooting medium consisting of half strength MS medium supplemented with 3 mg L⁻¹ IBA along with 0.1 mg L⁻¹ BAP with or without 1% wv⁻¹ activated charcoal. After four weeks of culture, shoots with roots were washed thoroughly to remove any trace of medium and were shifted to a Knop salt solution without any sucrose (Prasad *et al.*, 1975).

Plantlets with healthy shoots and roots were taken from Knop solution and washed thoroughly with a pinch of bavistin in sterile water. They were transferred to plastic pots with coco peat (Home Green Agri Private Limited) and sand in 1:1 combination and were covered with polythene bags to maintain high humidity and were watered on a daily basis. Polythene bags were perforated gradually for acclimatization and were transferred to greenhouse conditions.

All the data were analyzed by Analysis of Variance (ANOVA) followed by Duncan's multiple range tests using SPSS version 24.

Results and discussion

Explants inoculated on MS medium supplemented with BAP alone or in combination with KIN showed, darkening of the cut surface, bulging and velvety texture on the surface within two to three days after inoculation. The explants showed the development of a basipetal bud like structure which later swollen into globular structures called cormlets within four weeks. In the medium with lower concentration (1, 2 mg L⁻¹ BAP) of cytokinin, the explants initiated green callus. An average of four cormlets per explants were obtained on MS medium supplemented with 4mg L⁻¹ BAP within four weeks of culture (Table 1). The explants inoculated on MS medium supplemented with 4 mg L⁻¹ BAP and 1.5 mg L⁻¹ KIN developed maximum number of cormlets per explants (Fig. 2). About 90 % of explants showed four to eight cormlets production, after eight weeks of culture (Fig. 3A).

At the time of shoot regeneration, black surface of the cormlets developed white spotted appearance, which later produced numerous clumps of shoot buds, developing white and brownish mass of tissues within two weeks. An average of 19 shoot buds/

Table 1. Effect of different concentrations of BAP on MS medium in cormlet induction. Mean number of cormlets per explant after four and eight weeks of culture

Concentration of BAP mg L ⁻¹	No: of cormlets / explant after four weeks	No: of cormlets / explant after eight weeks
1	1.20±0.374 ^d	2.00±0.548 ^d
2	2.00±0.316 ^{cd}	3.60±0.400 ^c
3	3.20±0.374 ^b	5.20±0.374 ^b
4	4.40±0.245 ^a	7.40±0.510 ^a
5	2.80±0.374 ^{bc}	5.20±0.374 ^b

Values represent means ± SE of 10 replicates, those representing different letters in appropriate column are significantly different (ANOVA, P<0.05).

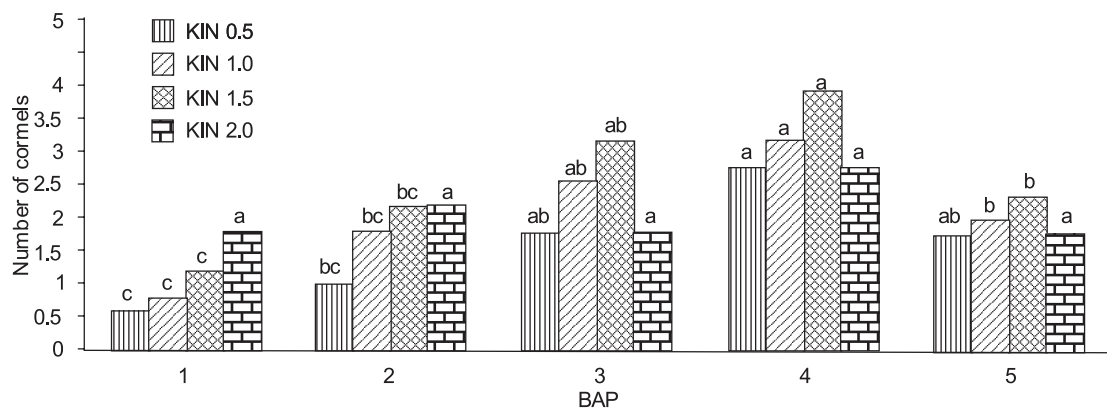


Fig. 2. Effect of different concentrations of BAP and KIN in different combinations on cormlet induction after four weeks of culture



Fig. 3. (A) Explant with eight to ten cormlets in 4 mg L⁻¹BAP along with 1.5 mg L⁻¹KIN.(B) Clumps of bud like white structures open out after six weeks resulted in thick, fleshy, white leafy shoot, later get transformed to cream, pale green (without glutamine). (C) One with glutamine. The path of regeneration from cormlets was exactly the same in both cormlets with and without glutamine. (D) Green fleshy leaves emerging from cormlets. (E) Leaf sheath and normal leaves developing after 12 weeks of culture. Bars:1 cm (A, B, C, D, E).

cormlets were observed after another two weeks on MS medium supplemented with 3 mg L⁻¹ IBA and 1.5 mg L⁻¹ BAP. These clumps of buds developed to fleshy, white leafy shoot, which then further turned to cream, pale green and finally green in colour. Bud development from cormlets without glutamine (Fig. 3B) and one with glutamine (Fig. 3C) showed similar path of developments. When it attained an average height of 2cm (Fig. 3D), normal shoots and leaves appeared within the clumps (Fig. 3E). On an average, 13 shoots/cormlet developed after six weeks of culture. Maximum number of shoot development was observed from explants inoculated on MS medium supplemented with 3 mg L⁻¹ IBA and 1.5 mg L⁻¹ BAP (Table 2).

The cormlets inoculated on MS medium supplemented with 3mg L⁻¹ IBA along with 1.5 mg L⁻¹ BAP and 1000 mg L⁻¹ glutamine showed larger cormlets. After four weeks of culture, an average

Table 2. Effect of different concentrations of IBA (1, 2, 3, 4 mg L⁻¹) and glutamine (500, 1000, 1500 mg L⁻¹) along with 1.5 mg L⁻¹ BAP on MS medium for shoot regeneration from cormlets. Mean number of shoot buds, sprouts and shoots were represented at fourth, and twelfth week of culture respectively.

Concentration of IBA mg L ⁻¹	Glutamine mg L ⁻¹	No. of shoot buds fourth week	No. of shoots twelfth week
1	-	5.40±0.92 ^d	1.80±0.37 ^d
2	-	13.40±0.92 ^b	9.00±0.70 ^b
3	-	19.80±1.35 ^a	13.60±1.50 ^a
4	-	9.00±1.00 ^c	5.00±0.70 ^c
3	500	22.60±1.20 ^b	17.00±1.41 ^b
3	1000	28.60±0.92 ^a	21.20±0.58 ^a
3	1500	17.40±1.28 ^c	11.40±1.03 ^c

Values represent means ± SE of 10 replicates, those representing different letters in appropriate column are significantly different (ANOVA, $P < 0.05$).

size of cormlet without glutamine was observed to be 1.14 cm in diameter while one with glutamine showed 2.70 cm in diameter. It was reported early that the role of proline and glutamine in improving callus induction and subsequent shooting in rice (Pawar *et al.*, 2015). Green *et al.* (1990) reported that the nature of nitrogen source can significantly affect the *in-vitro* responses of the explant. Organic nitrogen sources like amino acids proved to be beneficial for cell proliferation, increasing biomass of the explants as well as regeneration rate and helped to maintain high growth rate for a longer period than inorganic source like nitrates and ammonium (Gamborg *et al.*, 1968, Franklin *et al.*, 1991, Vasudevan *et al.*, 2004).

Shoot regeneration from cormlets inoculated on MS medium with glutamine showed higher number of shootbuds, sprouted shoot buds and shoots (Table 2). The path of regeneration of shoots from cormlets was exactly the same both with or without glutamine treatment. Histological study showed the presence of shoot meristems, emerging fleshy leaves within the cormlets (Fig. 4A, B and C). In the present study an average of 13 shoots developed per cormlet, and an addition of glutamine increased the cormlet size

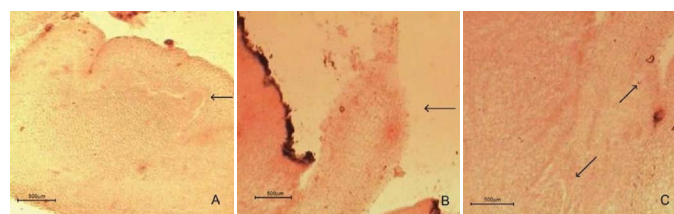


Fig. 4. (A) Longitudinal section of *in vitro* regenerated corm showed an apical meristem enclosed with fleshy leaves on either side which later gives rise to normal shoots, (B) elongated leaf sheath emerging from corm. (C) Lower portion of leaf sheath (upper arrow), inner to that internal apical meristem (lower arrow) for normal shoot appeared. Bars: 500 μm (A, B, C).

and produced an increased number of 21 shoots/cormlets. Similar results were reported by Aboshama (2011) where addition of 500 mg L⁻¹ glutamine to shoot regeneration medium showed highest number of shoot induction in *Buddleia globosa* and by Hamasaki *et al.* (2005) where 8 mM glutamine along with shoot induction medium indirectly increased endogenous IAA and iP levels resulting in increased explant competence for organogenesis in pineapple leaves.

The clumps of shoots obtained above were separated and grouped into ten, each with two plantlets and transferred to fresh medium with varying concentrations of BAP *viz.*, 3, 4, 5 or 6 mg L⁻¹ for shoot multiplication (Table 3). Higher concentration of BAP showed increase in shoots as well as leaf length. MS medium supplemented with 5 mg L⁻¹ BAP, showed significantly increased shoot as well as leaf numbers after four weeks of culture (Fig. 5A). When leafy shoot alone was transferred to shoot development medium, no response was observed, whereas the same with a little portion of corm showed normal shoot development. The rate of shoot multiplication was lower in IBA and BAP combination rather than BAP alone (Table 3). Hence in the present study Table 3. Effect of different concentration of BAP either alone or in combination with IBA on MS medium for shoot multiplication. Mean number of shoots, leaves and length of shoot (cm) after four weeks of culture.

Concentration (mg L ⁻¹)		Number of shoots	Number of leaves	Length of shoots (cm)
BAP	IBA			
3	-	5.80±0.37 ^c	12.20±0.97 ^b	3.14±0.09 ^b
4	-	7.20±0.37 ^b	16.20±1.46 ^a	3.30±0.15 ^b
5	-	9.00±0.44 ^a	19.00±1.00 ^a	4.02±0.16 ^a
6	-	2.40±0.51 ^d	7.60±1.50 ^c	3.90±0.18 ^a
5	0.5	0.50±0.298 ^c	1.75±1.03 ^c	1.45±0.83 ^b
5	1.0	4.00±0.548 ^b	13.80±0.917 ^b	3.540±0.248 ^a
5	1.5	6.60±0.510 ^a	18.60±1.030 ^a	3.660±.2657 ^a
5	2.0	1.00±0.447 ^c	3.20±1.463 ^c	1.760±0.719 ^b

Values represent means ± SE of 10 replicates, those representing different letter in appropriate column are significantly different (ANOVA, $P < 0.05$).

a higher cytokinin concentration was found to be better for maximum shoot multiplication. After attaining a height of 3-4 cm, shoots were transferred to rooting medium.

The shoots developed roots on half strength MS medium with 3 mg L⁻¹ IBA, 0.1 mg L⁻¹ BAP and 1% wv⁻¹ activated charcoal (Fig. 5B). Averages of three roots with 3.54cm length were obtained after four weeks of culture. Then the plantlets were transferred to Knop's solution. The plantlets adapted well in Knop solution and increased root number as well as root length. A maximum of six to eight roots/shoot having a length of 6-10 cm were observed with new leaves within four weeks (Table 4). Rooted plants were transferred to cocopeat and sand in 1:1 combination and showed

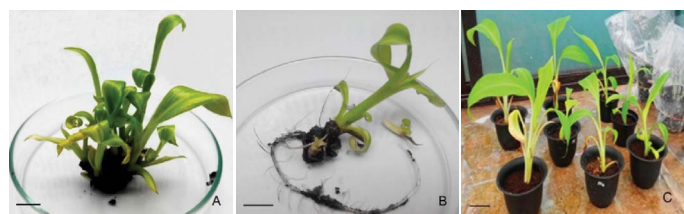


Fig. 5. (A) Multiple shoots obtained in MS medium with 5 mg L⁻¹ BAP (B) Rooting in half strength MS with high auxin to cytokine (IBA 3 mg L⁻¹, BPA 0.1 mg L⁻¹) along with activated charcoal. (C) Hardened plants in green house. Bars: 1 cm (A, B), 4 cm (C).

Table 4. Effect of activated charcoal and Knop's Solution on rooting. 3 mg L⁻¹ IBA along with 0.1 mg L⁻¹ BAP in half strength MS medium for root induction. NR- number of roots, LR- Length of the longest root was represented in cm. Results were recorded after fourth and eighth week of culture.

Concentration of PGHs IBA 3 mg L ⁻¹ + BPA 0.1 mg L ⁻¹	Activated charcoal 1% wv ⁻¹	Fourth week		Transferred to Knop's Solution eighth week	
		Number of roots	Length of longest root	Number of roots	Length of longest root
-	-	1.00 ± 0.31 ^b	1.16 ± 0.33 ^b	2.40 ± 0.24 ^c	3.14 ± 0.15 ^c
-	+	1.60 ± 0.51 ^{ab}	1.80 ± 0.46 ^b	4.20 ± 0.37 ^b	4.14 ± 0.18 ^b
+	-	1.20 ± 0.37 ^b	1.10 ± 0.33 ^b	3.40 ± 0.24 ^{bc}	3.76 ± 0.25 ^{bc}
+	+	3.00 ± 0.31 ^a	3.54 ± 0.20 ^a	6.40 ± 0.51 ^a	6.24 ± 0.34 ^a

Values represent means ± SE of 10 replicates, those representing different letters in appropriate column are significantly different (ANOVA, $P < 0.05$).

98% survival rate and weremaintained in greenhouse (Fig. 5C).

Most of the earlier reports on micropropagation of *Ensete*, used shoot tips from corm tissue as explants (Afza *et al.*, 1996, Negash *et al.*, 2000, Diro *et al.*, 2004). The source of shoot tip explant was either greenhouse grown plants or *in-vitro* germinated seedlings (Negash *et al.*, 2000). Availability of such explants was not easy since the species population is decreasing. Hence present study used inflorescence tip as explants.

Earlier report on clonal propagation of *Ensete* through shoot tip cultures showed an average of 1-4 cormlets per explant on MS medium with 1.5 mg L⁻¹ BAP and 1mg L⁻¹ KIN, within four weeks of culture (Mathew *et al.*, 1996). However inflorescence explant showed higher yield of cormlets with in a shorter period of four weeks. Even though high phenolic exudation retards responses, darken the medium, showed degradation and necrosis from the distal end of the explant, it helped in reducing contamination, development of both corms as well as healthy green leafy shoots. Similar to corm development, leafy shoots development was also basipetal. Earlier report on *E. ventricosum* showed normal shoot development required a little portion of corm (Birmeta *et al.*, 2004).

In the present study a single inflorescence may yield approximately more than 60 explants and contamination rate was also less or completely absent. An average of 21 shoots developed per cormlet indicating the potential of this method for conservation of this species.

Traditional vegetative propagation methods already published for this species (Karlsson *et al.*, 2015), reportedly took about nine months for sucker induction at the expense of 63 corms of two year old plants, buried under soil. A similar vegetative propagation method was reported by Simmonds (1966) in *E. superbum*, where, before flowering, hollowed corms were stuffed with soil and dung. Both found to be time consuming. The present study introduced an efficient micropropagation method using apical meristem in the inflorescence as explants, without harming its natural means of propagation or its habitat, thereby successfully contributing to the conservation of wild gene pool and therapeutic potentials of this rare, threatened rock banana - *E. superbum*.

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