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Control of Phenolic Compound Secretion and Effect of Growth Regulators for Organ Formation from *Musa* spp. cv. Kanthali Floral Bud Explants

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Abstract: The study was undertaken to examine banana cv. Kanthali floral bud apex as an alternative source material for *in vitro* propagation because huge number of explants die due to microbial contamination in case of shoot tip explants. Contamination free cultures were established by treating the floral bud explants with 0.1% $HgCl_2$ for 6 min. This study found that inflorescence tissues of experimental plant was almost contamination free but was high in phenolic compounds. Phenolic compounds secretion was successfully stopped by pre-soaking them in an antioxidant solution of 0.125% potassium citrate: citrate. After antioxidant treatment the floral bud explants were cultured on MS medium supplemented with different concentrations and combinations of BA+Kn+IAA/IBA+15%CW. Compact, white/greenish white callus was formed in different amount at all concentrations after 3 weeks of culture. All were again subcultured at same medium and after another 30-35 days at 2.0 mg L^{-1} BA+2.0 mg L^{-1} Kn+2.0 mg L^{-1} IAA+15%CW some callus showed embryogenic structure.

Key words: *Musa* spp. cv. Kanthali, floral bud apex, antioxidant, callus, somatic embryo

INTRODUCTION

Banana (*Musa* spp.) is the 4th largest food crop in the world and affects lives of 400 million people[1]. It is a nutritious fruit rich in carbohydrates and a good source of vitamins. *Musa* spp. cv. Kanthali (genome AAB) is a traditional table banana cultivar of Bangladesh[2]. The plant is only found in the southern part of the country and its population is continuously decreasing due to lack of commercial cultivation. Its production rate is relatively lower but the plant is more salt tolerant and disease resistant than other commercial cultivars.

The development of micropropagation techniques has been a major focus of *Musa* research during the past two decades and such techniques have now been well established[3,4]. Micropropagation of banana has been achieved using shoot tip[5] and from male floral apices[6]. Meristem culture for clonal propagation and virus eradication was done by Gupta[7]. There are also reports of somatic embryogenesis and regeneration in liquid medium[8-10]. Utilization of whole flowers, buds, ovary sections and inflorescence sections as primary explant source material has been reported[11-13]. Bunn and Dixon[14] demonstrated that adventitious shoot formation arises directly from the perianth or external ovary tissues in *Blandfordia grandiflora*, with this technique proving advantageous for rapid shoot regeneration with minimal explant material. This protocol is also useful for conservation of endangered species as it is a nondestructive method utilizing seasonal structures of the plant therefore allowing for the preservation of the mother plant[12]. This method of *in vitro* propagation demonstrates that many hundreds of clones can be obtained successfully from a single inflorescence of date palms[15].

For *in vitro* propagation of banana, bacterial contamination is a great problem. Although initially surface sterilization works, later on microbial contamination at the base of the explant is observed within 7 to 15 days after inoculation. Bacterial growth is also observed around the explants in the culture media. Huge number of explants is destroyed in the culture due to endogenous bacteria[16]. In this aspect, utilization of immature inflorescence tissue as explant material can favors minimal contamination rates compared to other tissues. Another problem of *in vitro* cultured explants, accompanied by darkening of culture medium has been attributed to phenolic compounds exuded from tissues and accumulating in the culture medium. This process is initiated by browning of the surface of plant tissues due to the oxidation of phenolic compounds resulting in the formation of quinones which are highly reactive and toxic to plant tissue[17]. Understanding the processes contributing to the oxidation of phenols and how these can be minimized when initiating explants is critical for successful *in vitro* culture.

However, high productivity in agriculture output has been mainly achieved through breeding programs and genetic manipulation. Therefore, present investigation was carried out to establish *in vitro* rapid clonal propagation of *Musa* spp. cv. Kanthali from its floral bud apices. The aims of the experimental design addresses the following aspects of *in vitro* development are:

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* That sterilization procedure can be developed which will optimize tissue survival *in vitro*.
* That antioxidant treatment minimizes phenolic exudation.
* That cytokinin and auxin ratios will facilitate tissue regeneration.
* That certain tissue types will respond to successful regeneration.

MATERIALS AND METHODS

Collection and preparation of explants: Banana cv. Kanthali (Genome AAB), a Cavendish type leading traditional cultivar of southern part of Bangladesh was the investigating subject. The source material used for culture establishment was banana floral bud explants. The plant materials were collected from a village named Amtola in Batiaghata Thana, Khulna and was very near from Khulna University campus.

Terminal floral apices of banana were collected from mature plants after they produced all possible hands. The terminal bud was cut from the peduncle and the bracts with their associated hands of male flowers were removed in a stepwise manner until they became too small to remove by hand. Working with a dissecting microscope, scalpel and forceps, the remaining bracts and minute hand of flowers were removed until the rounded growing point was exposed. The floral apex and approximately 1 cm long subtending peduncle tissue were excised.

Disinfection procedure: The surface sterilization procedure began with dissection of explant material into manageable units. Stem sections containing axillary buds and immature inflorescences were treated by initially removing the small leaflets and cleaning away surface detritus under running tap water for 1 to 2 min. A plastic vessel (130 mm x 320 mm x 120 mm) was used for treatments with sterilant solution. Sterilization was undertaken for 6 min using 0.1% (w/v) $HgCl_2$. Explants were transferred to a separate vessel for the washing phase in three changes of sterile distilled water.

Antioxidant treatment: The extracted buds were placed in petri dishes containing an antioxidant wash of 0.125% potassium citrate: citrate (K-C: C in a ratio of 4:1 w/w) solution. A concentrated stock of the antioxidant wash was filter sterilized (with 0.22-µM disposable filter) and frozen in 10 mL units until required. The concentrate was later thawed and further diluted with SDW to give the final 0.125% concentration. Petri dishes (90 mm x 14 mm) were filled with sufficient antioxidant solution to fully cover the explants. Peduncle sections were cut into discs under the antioxidant solution to minimize browning during initial preparation. Each explant was placed in a test tube containing 20 mL of media after five min in the antioxidant treatment.

Growth responses: After antioxidant treatment the floral bud explants of banana were cultured on MS solid medium supplemented with cytokinins, auxins and coconut water for initiating vegetative growth. After 3 weeks of culture compact, white/greenish white callus was formed more or less at all treatments. All were again subcultured at same medium and after another 30-35 days at a specific concentration some callus showed embryogenic structure but others remained unchanged. These were observed at the conclusion of this research and were not able to be further analyzed.

RESULTS AND DISCUSSION

The experiment was conducted at Plant Biotechnology Laboratory of Khulna University, Bangladesh during January to November 2005. One indigenous banana plant (*Musa* spp. cv. Kanthali) of Bangladesh was studied in order to establish suitable protocols for *in vitro* plant regeneration. In the present investigation *in vitro* growth responses of floral bud apices from mature plants was studied for large scale plant propagation.

For culture initiation all the experimental explants were cultured on MS medium supplemented with different concentrations of cytokinins and auxins for promoting the morphogenic responses. The results of the experiment are described as follows:

Sterilization procedure for explant: To overcome contamination problem surface sterilization of explants was done with 0.1% (w/v) $HgCl_2$ for different durations to assess the contamination percentages and viability of the explants used for *in vitro* culture.

Contamination free cultures with elegant survivability (100%) were achieved by treating the explants with 0.1% $HgCl_2$ for 6 min (Table 1).

Techniques applied for reducing phenolic compound secretion of explants: One of the most common problems associated with the *in vitro* establishment of many monocotyledonous and woody species is the deleterious effects of oxidized phenols[18,19]. The results of the detection of phenolic compounds experiment (Table 2-4) clearly indicate that *Musa* spp. cv. Kanthali has phenolic compounds present in inflorescence tissue. This experiment provides a simple technique for detecting the presence of phenolic compounds in banana inflorescence tissue. This procedure assists in early detection of phenols[20] and assists in preparation of explant source material to reduce injury associated with phenolic oxidation. George[21] describes an antioxidant as an electron donor (reducing agent) which

Table 1: Standardization of $HgCl_2$ treatment period for surface sterilization of the explants

Treatment duration (min.) with 0.1% $HgCl_2$	Number of explants	Rate of contamination (after days of treatment)					Percentage of contamination free explants after 15 days
		3	6	9	12	15	
1	10	-	5	7	8	10	-
2	10	1	4	6	6	7	30
4	10	-	1	1	3	3	80
6	10	-	-	-	-	-	100
8	10	-	-	-	-	-	100*
10	10	-	-	-	-	-	100**
12	10	-	-	-	-	-	100***
15	10	-	-	-	-	-	100****

"-" indicates no contamination
"*" indicates explant death due to tissue killing
(* = 5-25%; ** = 26-50%; *** = 51-75% and **** = 76-100%)

Table 2: Antioxidant treatments

Treatment Number	Treatment	Volume of antioxidant treatment (ml)
1	Exposed to air, cut on wet filter paper	0
2	Cut in petri dish plus H_2O (SDW)	100
3	Cut in petri dish plus H_2O (reverse osmosis)	100
4	Cut in K-C: C (0.125% w/v)	100
5	Cut in K-C: C (0.125% w/v) + L-cysteine	100
6	Cut in K-C:C (0.125% w/v) + L-cysteine + Ascorbic acid	100

Table 3: egree of tissue discoloration after incubation in NaOH solutions for *Musa* spp. cv. Kanthali

Section type	Control (SDW)	0.001M NaOH	0.01M NaOH	0.1M NaOH	1M NaOH
Peduncle sections	O	O	+	+ +	+ + +
Pedicel sections	O	O	+	+ +	+ + +
Bract tissue	O	O	+	+ +	+ +

"O" no discoloration
"+" low discoloration
"+ +" medium discoloration
"+ + +" high (darkly stained)

inhibits the oxidation of labile substrates. The antioxidant compounds utilized in the experimental work in this chapter were selected because they have been used successfully in the past to delay browning in other arborescent monocotyledonous species[18]. George[21] details the use of citric acid and ascorbic acid combinations to delay browning. The successful prevention of browning in explants of *Musa textilis* by using a mixture of ascorbic acid, citric acid and cysteine are reported by Mante and Tepper[22]. The behavior of the citrate in citric acid works as a chelating agent bonding to ions responsible for activating polyphenol oxidative enzymes[21]. Ascorbate behaves as a reducing agent and is converted to dehydro-ascorbic acid[20]. Ascorbate is able to scavenge oxygen radicals produced when tissue is damaged and therefore cells are protected from oxidative injury. Oxygen radicals are attributed to exacerbating oxidative injury. Antioxidants containing citrate and ascorbate reduce browning of tissue by detoxifying these free radicals. The results provide evidence that K-C: C combination is a useful antioxidant for explant preparation for *Musa* spp. cv. Kanthali.

Musa spp. cv. Kanthali stems are susceptible to tissue browning and elimination or minimization of this process is an essential prerequisite to successful culture establishment. Therefore identification of a suitable treatment to minimize tissue browning in the explant source material of *Musa* spp. cv. Kanthali will be the focus of this chapter. The specific aim of this component of the study was to:

* Research methods for reducing phenolic induced injury in *Musa* spp. cv. Kanthali during explant preparation with particular emphasis on the use of appropriate antioxidant treatments.
* Detection of phenolic compounds using sodium hydroxide (NaOH)
* Detection of phenolic leakage was tested in the study plant *Musa* spp. cv. Kanthali. Flowering stems of *Musa* spp. cv. Kanthali were collected from a village named Amtola in Batiaghata Thana, Khulna for assessment. Concentrations of sodium hydroxide (NaOH) were made at rates 1 M, 0.1 M, 0.01 M and 0.001 M to treat the various plant tissues. NaOH oxidizes phenols which causes darkening of affected or damaged tissue[20]. Peduncle sections and pedicel slices of *Musa* spp. cv. Kanthali were cut in 2 mm thick discs and submerged in petri dishes containing the concentrations of NaOH for 5 min. Similarly, bract tissue was cut into squares approximately 15 mm x 15 mm and also placed in the NaOH solutions. Observations were made of any browning on the surface or cut edges of the various plant tissue to assess if phenolic leakage had occurred.

Table 4: Relative browning of disc sections of *Musa* spp. cv. Kanthali treated with antioxidants over a two hour period

Time (min)	Treatment Number					
	1	2	3	4	5	6
0	+ + +	+ +	+	O	O	O
6	+ + +	+ + +	+ +	O	+	O
30	+ + +	+ + +	+ + +	O	+	+
60	+ + +	+ + +	+ + +	O	+ +	+ +
120	+ + +	+ + +	+ + +	O	+ + +	+ + +

"O" no oxidation
"+" low oxidation
"+ +" medium oxidation
"+ + +" high oxidation

Treatment numbers:
1 → Exposed to air, cut on wet filter paper.
2 → Cut in petri dish plus H_2O (SDW)
3 → Cut in petri dish plus H_2O
4 → Cut in K-C: C (0.125% w/v)
5 → Cut in K-C: C (0.125% w/v) + L-cysteine
6 → Cut in K-C: C (0.125% w/v) + L-cysteine + ascorbic acid

Table 5: Effect of different concentrations of BA in combination with Kn, IAA and 15% CW for callus induction from banana floral bud explant. There were 10 explants for each treatment and data were taken after 3 weeks of culture

Growth regulators conc. (mg L^{-1}) (BA + Kn + IAA + 15% CW)	Degree of callus formation	Callus character
0.5 + 0.5 + 0.5	+	Compact, hard, white
0.5 + 1.0 + 1.0	+	Compact, hard, white
0.5 + 2.0 + 2.0	+ +	Compact, hard, greenish white
1.0 + 0.5 + 0.5	+	Compact, hard, white
1.0 + 1.0 + 1.0	+ +	Compact, hard, greenish white
1.0 + 2.0 + 2.0	+ +	Compact, hard, white
2.0 + 0.5 + 0.5	+ +	Compact, hard, white
2.0 + 1.0 + 1.0	+ + +	Compact, hard, white
2.0 + 2.0 + 2.0	+ + +	Compact, hard, white

"+ + +" indicates excellent degree of callus
"+ +" indicates good degree of callus
"+" indicates poor degree of callus

Table 6: Effect of different concentrations of BA in combination with Kn, IAA and 15% CW for callus induction from banana floral bud explant. There were 10 explants for each treatment and data were taken after 3 weeks of culture

Growth regulators conc. (mg L^{-1}) (BA + Kn + IBA + 15% CW)	Degree of callus formation	Callus character
0.5 + 0.5 + 0.5	+	Compact, hard, white
0.5 + 1.0 + 1.0	+	Compact, hard, white
0.5 + 2.0 + 2.0	+ +	Compact, hard, greenish white
1.0 + 0.5 + 0.5	+	Compact, hard, white
1.0 + 1.0 + 1.0	+ +	Compact, hard, greenish white
1.0 + 2.0 + 2.0	+ +	Compact, hard, white
2.0 + 0.5 + 0.5	+ + +	Compact, hard, white
2.0 + 1.0 + 1.0	+ + +	Compact, hard, white
2.0 + 2.0 + 2.0	+ +	Compact, hard, greenish white

"+ + +" indicates excellent degree of callus
"+ +" indicates good degree of callus
"+" indicates poor degree of callus

Antioxidant experiment: Pedicel and peduncle disc sections from *Musa* spp. cv. Kanthali were collected from a mature flower stem and treated with various antioxidant solutions. Disc sections were selected as they have a large surface area and have been shown to be prone to oxidation (Table 2).

A stock solution of potassium citrate and citrate (K-C: C) was made up using 1 g K-C and 0.25 g citrate and dissolved in 10 mL of SDW. The concentrate was then diluted and used at a final concentration of 0.125%. For treatments 5 and 6 0.02 g L^{-1} L-cysteine HCl was added to the 0.125% solution of K-C: C and 0.25 g L^{-1} ascorbic acid was added to treatment 6. One hundred milliliters of the various solutions were used to fully cover the disc sections with the control treatment cut on filter paper and exposed to air. All other material was cut under the various treatments to avoid exposure to the air. The prepared disc sections were placed onto water agar petri dishes and results were recorded at time at intervals of 0, 7, 30, 60 and 120 min. Observations of the extent of browning were recorded.

Detection of phenolic compounds using sodium hydroxide (NaOH): Pedicel and peduncle sections produced a large degree of discoloration after having been sliced into discs and placed in the NaOH. The

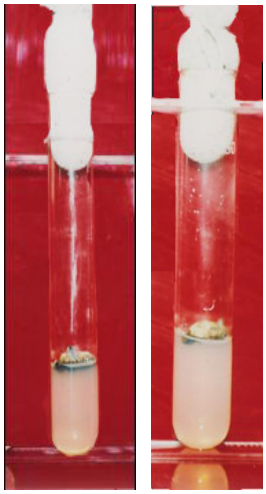
bract tissue of *Musa* spp. cv. Kanthali developed a distinct green/brown line, approximately 2 mm wide around the cut edges (Table 3).

Antioxidant experiment: All cut surfaces in the control appeared to oxidize rapidly once exposed to air as evidenced by tissue browning. Subsequently all other tissues were prepared under each of the antioxidant treatments. Pedicel sections showed similar rates of browning to excised flower bud material when exposed to air without antioxidant treatment. Treatments 4, 5 and 6 initially reduced browning of the disc sections and after 2 hrs, treatment 4 (K-C: C) was visually better than the other 2 antioxidant treatments (Table 4).

Potassium citrate-citrate combinations as antioxidant treatments for excised tissue: All tissues initiated into culture were treated with the K-C: C treatment as it proved to be the best treatment type from experimental results. The antioxidant treatment reduced browning in all tissue types after 24 hrs in culture. The cut surfaces and any damaged areas of untreated tissue (particularly peduncle sections) turned brown within 15 min after the excision. These explants continued to oxidize under culture conditions and were completely brown after 1 hr and were subsequently discarded.

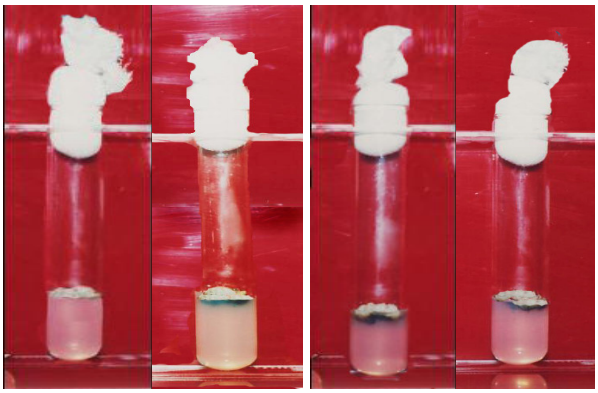
After 3 weeks of culture the phenolic leakage in treated tissues was minimized and in most cases controlled. Many of the explants had remained pale while others had started showing signs of greening. Some minor staining of the media was evident in some explant tissues predominantly in peduncle sections. The results from this study indicate the browning phenomenon in *Musa* spp. cv. Kanthali tissue can be greatly reduced by pre-soaking of explants in antioxidant solution of 0.125% (w/v) potassium citrate and citrate prior to culture. Also, incubation in the dark for the first 1 week may arrest the rate of tissue browning by slowing the enzymatic activity responsible for tissue oxidation. Frequent subculturing to fresh medium may also assist so that toxic phenolic compounds do not hinder the activity of plant growth regulators on tissues. The combined effects of the treatments outlined above proved beneficial to explant survival *in vitro* of *Musa* spp. cv. Kanthali inflorescence tissue (Fig. 1).

Organ formation from floral bud explants: Floral bud apices of banana (*Musa* spp. cv. Kanthali) were isolated aseptically. Then after successful sterilization and antioxidant treatment they were cultured on MS medium supplemented with different cytokinins, auxins and coconut water for initiating vegetative growth (Table 5).



A B

Fig. 1: Comparison between untreated (A) and treated (B) floral bud apex explants for excessive phenolic compound secretion



A B

Fig. 2: Excellent degree of callus formation from banana floral bud explants on MS medium supplement with 2.0 mg L^{-1} BA + 1.0 mg L^{-1} Kn + 1.0 mg L^{-1} IAA + 15% CW (A) and 2.0 mg/l BA + 2.0 mg L^{-1} Kn + 2.0 mg L^{-1} IAA + 15% CW (B)

Effect of different concentrations and combinations of BA, Kn, IAA and 15% CW for callus induction: Floral bud explants of banana were cultured on MS solid medium supplemented with different concentrations and combinations of BA, Kn, IAA and 15% coconut water for initiating vegetative growth. After 3 weeks, cultures showed enlargement of the floral primordial and compact, whitish/greenish white callus was formed more or less at all treatments. But at MS + 2.0 mg L^{-1} BA + 1.0 mg L^{-1} Kn + 1.0 mg L^{-1} IAA + 15% CW and MS + 2.0 mg L^{-1} BA + 2.0 mg L^{-1} Kn + 2.0 mg L^{-1} IAA + 15% CW excellent degree of callus was formed. And very poor degree of callus was formed at MS + 0.5 mg L^{-1} BA + 0.5 mg L^{-1} Kn + 0.5 mg L^{-1} IAA + 15% CW and MS + 1.0 mg L^{-1} BA + 0.5 mg L^{-1} Kn + 0.5 mg L^{-1} IAA + 15% CW.

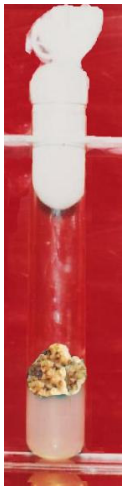


Fig. 3: Embryogenic callus formation from floral bud explant of banana on MS + 2.0 mg L^{-1} BA + 2.0 mg L^{-1} Kn + 2.0 mg L^{-1} IAA + 15% CW

Effect of different concentrations and combinations of BA, Kn, IBA and 15% CW for callus induction: The floral bud explants of banana were again cultured on MS solid medium supplemented with different concentrations and combinations of BA, Kn, IBA and 15% coconut water for observation of their vegetative growth. After 3 weeks, cultures showed enlargement of the floral primordial and compact, whitish/greenish white callus was formed more or less at all treatments just like previous treatment. But at MS + 2.0 mg L^{-1} BA + 0.5 mg L^{-1} Kn + 0.5 mg L^{-1} IBA + 15% CW and MS + 2.0 mg L^{-1} BA + 1.0 mg L^{-1} Kn + 1.0 mg L^{-1} IBA + 15% CW excellent degree of callus was formed (Table 6).

Subculture for somatic embryo like structure development: All the callus were again subcultured at same medium and after another 30-35 days at 2.0 mg L^{-1} BA + 2.0 mg L^{-1} Kn + 2.0 mg L^{-1} IAA + 15% CW some callus showed embryogenic structure. The embryogenic callus showed several developed embryos on the surface. However, conversion of these embryos into plants did not occur, probably due to a lack of development of the shoot apical meristem. Similar results were obtained by Lee *et al.*[23] and Grapin *et al.*[24] with banana somatic embryos and Nickle and Yeung[25] with *Daucus carota* L (Fig. 2).

More detailed studies should be developed aiming to obtain banana somatic embryos of higher quality, which can efficiently convert into plants. Somatic embryo quality can be improved with optimization of parameters like the type and concentration of plant growth regulators, time in induction medium and maturation treatments. Somatic embryo quality is essential for high somatic embryo conversion rates (Fig. 3).

CONCLUSION

This study examined the application of micropropagation protocols to assist germplasm conservation of a traditional cultivar table banana [*Musa* spp. cv. Kanthali (Genome, AAB)] of Bangladesh. This has implications for commercial explant production in large scale as it generally produce 5-6 suckers from a mature mother plant per year. For *in vitro* propagation of banana, bacterial contamination is a great problem. So, the aim was to investigate the floral bud apices as an alternative explant source material because it favors minimal contamination rates compared to other tissues. Key factors investigated in this study includes sterilization technique to establish contamination free culture, control of phenolic exudates in explant preparation and the selection of appropriate growth regulator levels to achieve successful *in vitro* regeneration.

One of the most commonly encountered problems in *in vitro* culture establishment is the contamination by microbial contaminants. One hundred percent contamination free culture was established by soaking the floral bud explants in 0.1% $HgCl_2$ for 6 min followed by several washes in sterile water obviated the need to develop extensive and complicated surface sterilization protocols.

This study found that inflorescence tissues of experimental plant were high in phenolic compounds. The oxidation of tissues was severe and proved deleterious to all tissues in the initial stages of explant preparation. So, for reduction of phenolic compounds explants were pre-soaked in antioxidant solution of 0.125% (w/v) potassium citrate: citrate. Then they were placed in dark for 1 week so that rate of tissue browning was arrested. The effect of the treatments outlined above proved beneficial to explant survival *in vitro* of *Musa* spp. cv. Kanthali inflorescence tissue.

For successful explant establishment, a wide range of cytokinin and auxin combinations were investigated. The floral bud apices were cultured for 3 weeks on MS basal medium supplemented with various concentrations and combinations of cytokinins, auxin and additives. Hard, compact, white/greenish white callus was formed in different amounts at all concentrations. All were again subcultured at same medium and after another 30-35 days at 2.0 mg L^{-1} BA + 2.0 mg L^{-1} Kn + 2.0 mg L^{-1} IAA + 15% CW some callus showed embryogenic structure. Present investigations have thus shown that the economically valuable *Musa* spp. cv. Kanthali could be regenerated *in vitro* via somatic embryogenesis. The protocol developed will be useful for rapid *in vitro* propagation of the species and also for the subsequent genetic manipulation studies.

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Abbreviations

µM	Micromole
BA	6-Benzyl adenine
CW	Coconut water
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	Kinetin (6-furfuryl amino purine)
NAA	α-Napthaleneacetic acid
p^H	Negative logarithm of Hydrogen ion (H^+)
v/v	Volume per volume
w/v	Weight per volume

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