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IN VITRO REGENERATION PROTOCOL OF BANANA CV. BHIMKOL (*MUSA BALBISIANA,* BB GENOME) THROUGH EMBRYOGENIC CELL SUSPENSIONS (ECS) FROM IMMATURE MALE FLOWERS

Limasunep Longkumer *
Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, Assam, India
E-mail: limasuneplkr@gmail.com
Orcid: https://orcid.org/0000-0002-8345-992X

Abhik Gogoi
Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, Assam, India

Neha Maske
Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, Assam, India

Salvinder Singh
Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, Assam, India

Abstract

Musa balbisiana is the diploid BB genome of *Musa* species belonging to *Musa*ceae family. The cultivar Bhimkol (*Musa balbisiana* cv. Bhimkol) is found all over North-Eastern region of India. Although considered as a wild diploid species, this particular banana cultivar has been utilised as an important food in these parts of the country. In Assam, this fruit is utilised especially utilized as a nutrition supplement for infants. The banana pulp of Bhimkol is processed and marketed locally as a baby food nutrition supplement powder such as Bhimshakti and Bhimvita. This is one of the most important reasons which this banana cultivar a very important crop in this part of the country. There is a need for generation of a novel and an efficient *in vitro* regeneration protocol of *M. balbisiana* cv. Bhimkol using the male inflorescence of Bhimkol for carrying out any genetic improvement studies. Hence, this research, we report an efficient as well as reproducible *in vitro* regeneration protocol using the embryogenic cell suspension (ECS) cultures developed from the male inflorescence of *M. balbisiana* cv. Bhimkol.

Keywords: *Musa balbisiana* cv. Bhimkol, *in vitro* regeneration, embryogenic cell suspension (ECS).

1. INTRODUCTION

Bhimkol (*Musa balbisiana,* BB genome) is a wild type and also one of the most popular seeded type of banana, which are under large scale cultivation in North Eastern India. However, Bhimkol has not been divided into subspecies because of its low variability. Simply put, the wild accessions are referred to as types, which are characterized by the region or locale where they were gathered.

It is crucial to bring up Bhimkol and Elavazhai while discussing wild forms of *M. balbisiana* because they are commercially used by the people of Assam, as well as in the Western Ghats region of Karnataka and Kerala. In Assam, Bhimkol is no longer regarded as wild and has become a common household variety over time. Bhimkol is most extensively grown in Assam in northeastern India, with lesser cultivation occurring in West Bengal, Arunachal Pradesh, Nagaland, Meghalaya, and other states. There is little to no variation among Bhimkol, according to a thorough survey and analysis of diversity using morphological and molecular characteristics. This is because Bhimkol has a high barrier to fertilization and pollination, and the seed development is never fully completed. The ovules grow into seeds, but they lack a fully formed embryo and endosperm (Uma *et al*., 2006).

Basically, banana is a vegetatively propagated crop and they are propagated from the swords or suckers which arises near the rhizome of the mother plant. This makes it difficult to initiate any crop improvement program in banana since conventional breeding is complicated in banana and only few diploid cultivars produce viable pollen. It may also be noted that most commercial cultivars are triploid producing flowers which are both male as well as female sterile (Novak *et al*., 1990). Besides conventional breeding, improvement of traits in banana can be carried out by genetic engineering methods. However, for the application of genetic engineering in order to improve the desirable traits by introgression of a foreign gene or knock-out of an undesirable trait, it is essential to have an efficient and reproducible tissue culture protocol in banana. Although, *in vitro* regeneration of banana was possible utilising different explant sources such as leaf sheaths, corm sections and embryonic cell suspension cultures developed from highly proliferating shoot tips and male flowers, much of these has been done only in the triploid variety of banana. By keeping this in view and realising commercial importance of the diploid variety of banana (*cv*. Bhimkol) we have designed and optimized highly efficient protocol for *in vitro* regeneration of *Musa balbisiana cv.* Bhimkol through the somatic embryogenesis by embryogenic suspension cultures (ECS) developed from the immature male flowers.

2. MATERIALS AND METHODS

Plant Material and Explant

Male flowers of Bhimkol have been collected from farmers' fields of Jorhat district, Assam. The outer bracts of the spadix were removed, leaving about 10 cm of the inner core of the inflorescence. The primed inflorescence of Bhimkol were then transferred to a laminar air flow cabinet where they have been surface sterilized twice with 70% (v/v) ethanol. Using a sterile surgical blade (Glass van No. 10, India), the immature male flowers (the inner bracts) from the apical dome up to 15 inner bracts were separated and used to generate embryogenic calli under the laminar air flow cabinet. After that, embryogenic cell suspensions (ECS) were started and established using the embryogenic calli.

Plant Nutrient Media

Modified MS medium (Murashige and Skoog, 1962) modified "SH medium (Schenk and Hildebrandt, 1972) supplemented with different concentrations of growth hormone i.e., 1 mgL^{-1} to 5 mgL^{-1} of 2, 4-D dichlorophenoxyacetic acid (2,4-D), 100 μgL^{-1} to 300 μgL^{-1} of zeatin, 50 mgL^{-1} to 150 mgL^{-1} of glutamine, 2 mgL^{-1} to 6 mgL^{-1} of 6-Benzyl amino purine (BAP) and 1 mgL^{-1} to 3 mgL^{-1} of Indole-3- butyric acid (IBA) were utilised in this research. The stock solution of" different constituents of the MS medium was prepared as described by Murashige and Skoog, 1962. Major (macro) inorganic elements were synthesized in a stock solution at a concentration of 10X, whereas small (micro) inorganic elements as well as Fe-EDTA (Iron-ethylendiaminetetraaceticacid) were prepared at 100X concentration, while vitamin and "growth regulators were prepared at concentration of 1 mgL^{-1}. The different types of nutrient media used for the establishment of ECS are 1. Callus induction medium (CIM), a modified form of MS medium" (semi solid); 2. Suspension medium for ECS (M2), a modified MS medium (suspension); 3. Somatic embryo maturation medium (M3), modified SH medium (semi solid); 4. Germination medium for somatic embryos (M4), modified MS medium (semi solid); 5. Shoot elongation medium (M5), modified MS medium (semi solid) and 6. Rooting medium (R1), modified MS medium (semi solid), from *in vitro* regenerated shoots. A synthetic gelling agent, Phytagel was added for making semi-solid medium whenever required followed by heat sterilization in an autoclave at 15 PSI (12 °C) for 20 minutes. The sterilized medium was cooled to 50 to 55°C followed by addition of specific filter sterilized growth regulators according to type and need of media.

Induction of Embryogenic Calli

Using CIM nutritional "medium supplemented with 1 mgL^{-1} IAA, 1 mgL^{-1} NAA, and varying doses of 2, 4-D, namely 1 mgL^{-1}, 2 mgL^{-1}, 3 mgL^{-1}, 4 mgL^{-1}, and 5 mgL^{-1}, first 15 inner bracts are the juvenile male flowers from the apical dome. The cultures were kept in dark at 26±2°C for approximately five to six months in order to" induce callus. They were frequently sub-cultured on the same medium composition until embryogenic calli were seen.

Initiation and Maintenance of Embryogenic Cell Suspension (ECS)

ECS cultures have been initiated from six months old embryogenic calli. A portion of pro-embryogenic calli was used to establish ECS in a sterile 100 mL conical flask filled with 15 mL of M2 suspension medium supplemented with 1 mgL^{-1} 2, 4-D; 10 mgL^{-1} ascorbic acid and Zeatin at different concentrations such as 0.100 mgL^{-1}, 0.150 mgL^{-1}, 0.200 mgL^{-1}, 0.250 $mgL^{-1}$and 0.300 mgL^{-1}. The suspension cultures were maintained in an incubator shaker (Scigenics Biotech) under dark conditions with a circular shaking motion of 80 rpm at 25 ± 2 °C. Frequent sub-culturing was done by replacing old medium with fresh medium without harvesting the cells to increase the cell biomass and to remove phenolic substances secreted by the cells. After one month of culture, 5mL of settled cell volume has been transferred to 100mL conical flasks containing fresh 20 mL of M2 suspension medium for further cell proliferation. Somatic embryos have been observed after eight

months of the suspension cultures. The samples were routinely observed under stereoscopic microscope to check different morphological structures of the somatic embryos.

Maturation of Somatic Embryos

After eight months "of ECS cultivation, 5 mL of packed cell volume (PCV) from the ECS" were placed onto a filter paper with the use of a vacuum filter unit (ROCKYVACTM, Tarsons). The filter sheets with the somatic embryos were moved over a 90 mm sterilized petri-plate containing 25 mL of modified SH (Schenk and Hildebrandt, 1972) medium (M3) supplemented with Glutamine at varied concentration viz. 50 mgL^{-1}, 100 mgL^{-1}, 150 mgL^{-1} for somatic embryos maturation. For one to two months, these cultures were kept in a dark environment at 26 ± 2 °C.

Regeneration of Somatic Embryos

Onto somatic embryo germination medium (M4) supplemented with BAP at several dosages, such as 1 mgL^{-1}, 2 mgL^{-1}, 3 mgL^{-1} and 4 mgL^{-1}, the matured somatic embryos were placed. After a month had passed since the somatic embryos had begun to germinate, each shoot was moved separately onto modified "MS medium, which was supplemented with different amounts of BAP (M5), specifically 2 mgL^{-1}, 4 mgL^{-1} and 6 mgL^{-1} BAP, to promote shoot elongation. For two months, the cultures were sub-cultured every two weeks. The cultures were then kept at 25±2 °C with 16 hours of light (7000 lux) and 8 hours of dark photoperiod with" 80% relative humidity.

Rooting of the *in vitro* Regenerated Shoots

The rooting media (R1) mixed with varying concentrations of IBA, such as 1 mgL^{-1}, 2 mgL^{-1}, or 3 mgL^{-1}, was used to transfer "the in vitro regenerated shoots. For root induction, the cultures were kept at 25 ± 2°C with 80% relative humidity and 16 hours of light (light intensity of 7000 lux) and 8 hours" of dark photoperiod.

Hardening of *in vitro* Regenerated Bhimkol Plantlets

To obtain removal of "the nutrient media that was sticking to the roots, Bhimkol plantlets with both shoots and roots that had grown *in vitro* were removed from the culture container and cleaned under running water. After that, the plantlets were moved into" a potting mixture with a ratio 1:1:1 (w/w/w) sand: soil: vermicompost. These plantlets were transferred to Phytotron Unit for primary and secondary hardening followed by regular observation of morphological and vegetative growth of the plantlets.

3. RESULTS AND DISCUSSION

Standardization of *in vitro* Regeneration Protocol for *M. balbisiana* cv. Bhimkol

Mass "micropropagation and the creation of cellular tools for genetic enhancement (genetic transformation and protoplast fusion) were the primary objectives of somatic embryogenesis technologies when they were initially established. These methods rely on the induction of tissue dedifferentiation and the production of embryogenic tissue (callus) through the use of synthetic

growth regulators, or auxins. The foundation for development of ECS is provided by the callus. Plants recover and embryos are created from these suspensions. Banana plants already employ ECS for protoplast fusion and genetic change. In the case of altered plants, the problem of hybrid plants that occurs when using shoot tips as starting material is avoided because plants regenerated from an ECS frequently begin from a single" cell.

Zygotic "embryos (Cronauer and Krikorian, 1988, Escalant and Teisson, 1989), rhizome slices and leaf sheaths (Novak et al., 1990), and immature male/female flowers are the four methods that have been tested on banana plants (Lopez *et al*., 2022; Escalant *et al*., 1994; Grapin *et al*., 1996) as well as proliferating meristem cultures (scalps) (Dhed'a *et al*., 1991, Schoofs *et al*., 1997). Among these four different types of explants reports suggest that ECS are mostly initiated from immature male flowers or scalps (Lopez *et al*., 2022 and" Tripathi *et al*., 2012). In recent investigation, *in vitro* propagation of Bhimkol through ECS was not reported earlier, although Bhimkol has been extensively cultivated in Assam and other North-eastern states of India. As "an efficient and reproducible *in vitro* regeneration protocol is essential to carry out any genetic improvement in Bhimkol either through genome engineering and/or transgenics research. Hence, in recent research, we developed an *in vitro* regeneration system in Bhimkol through embryogenic cell suspension (ECS) from immature" male flowers.

Induction of Embryogenic Calli from Male Flowers of Bhimkol

Embryogenic calli have been induced by the male flowers of Bhimkol (Figure 1a) in modified MS semi-solid media (CIM) "supplemented with different concentrations of 2, 4-D (Table I). Among the treatments 4 mgL^{-1} 2, 4-D showed highest percentage of embryogenic calli" induction (9.31%) from male flowers after six months of culture (Figure 1b and 1c). This was followed by 5 mgL^{-1} 2, 4- D, that embryogenic calli induction frequency of 8.71% has been recorded after six months of culture. However, the frequency of embryogenic calli induction in 2 mgL^{-1} and 3 mgL^{-1} 2,4-D showed 4.05% and 6.21%, respectively. The least frequency (3.60%) for induction of embryogenic calli was observed in 1 mgL^{-1} 2, 4- D (Table I). Immature male flowers of banana cv. Bhimkol exude phenolic substances within a week of culture leading to discolouration and darkening of the medium neat the base of the explants. To avoid oxidation of polyphenolic substances, repeated sub-culturing was done after every fourteen days of culture. It was observed that the explants started to swell and increase in size after thirty days of culture, indicating the mitotic activity of the explants. Yellow globular calli were observed after three months of culture, which further developed into yellowish white coloured embryogenic calli after six months of culture (Figure 1c). Yellowish white embryogenic calli "has been found to be best for initiation and establishment of embryogenic cell suspension cultures for Bhimkol. The days to induce the embryogenic calli from immature male" flowers of *Musa acuminata* cv. Mas" (AA) was around six months after culture (Jalil *et al*., 2003).

Using synthetic auxin like 2, 4-D "for the initiation of embryogenic calli gave better results as compared to other synthetic auxins like NAA, IAA (Evans *et al*. 1981). 2, 4-D was considerably

used for the induction of embryogenic" "calli from male flowers of *Musa acuminata* cv. Mas (AA) (Jalil *et al*., 2003). It was also observed that after a certain threshold, increasing concentration of 2, 4-D reduces the induction of" embryogenic calli (Karintanyakit *et al*., 2011; Strosse *et al*., 2006). In recent research, we also seen that CIM supplemented with 5 mgL^{-1} of 2, 4-D reduces frequency of embryogenic calli induction to 8.71% as compared to the CIM supplemented with 4 mgL^{-1} 2, 4-D which resulted in a frequency of 9.31% after six months of culture (Table I).

Table I. Effect of 2, 4-D concentration for the induction of embryogenic calli from male flowers of Bhimkol in callus induction medium (CIM)

Treatment (2, 4-D)	No. of replicates	Immature male flowers inoculated	No. of explant forming embryogenic calli (after 6 months)	Percentage of embryogenic calli induction (%)
T1 (1 mg L^{-1})	5	145	5± 0.167	3.60
T2 (2 mg L^{-1})	5	141	6 ± 0.163	4.05
T3 (3 mg L^{-1})	5	145	9 ± 0.180	6.21
T4 (4 mg L^{-1})	5	140	13 ± 0.153	9.31
T5 (5 mg L^{-1})	5	139	12 ± 0.133	8.71

Establishment of Embryogenic Cell Suspension (ECS)

Embryogenic "cell suspension was initiated utilising the embryogenic calli induced from the male flowers of Bhimkol by transferring the calli in modified MS liquid media M2 supplemented with different concentrations of zeatin viz. 0.100 mgL^{-1}, 0.150 mgL^{-1}, 0.200 mgL^{-1}, 0.250 mgL^{-1} and 0.300 mgL^{-1} (Table II). The initiation and proliferation of ECS was observed after three months of culture in M2 medium. Highest percentage (50%) of" ECS establishment has been seen in M2 medium supplemented with 0.250 mgL^{-1} of zeatin (Figure 1d) followed by 0.300 mgL^{-1} zeatin which showed 45% of ECS established from embryogenic calli after eight months of culture. However, 0.200 mgL^{-1} and 0.150 mgL^{-1} of zeatin showed 40% and 31.58% of ECS establishment from embryogenic calli after eight months of culture, respectively. The lowest percentage (27.78%) of ECS was established in 0.100 mgL^{-1} of zeatin from embryogenic calli.

The creation of somatic embryos, a prerequisite for the establishment of an effective in vitro regeneration system in Bhimkol, was most likely when embryogenic cell suspension was established and maintained. The best explants for starting embryogenic cell suspension cultures (ECS) in Bhimkol were found to be friable yellowish white embryogenic calli from six-month-old cultures. After three weeks of culture with frequent sub-culturing of the cell suspensions, it was observed that the cells proliferated by producing fine aggregates of dividing embryogenic cells which were yellowish white in colour, thereby leading to an increase in embryogenic cell density. The homogenous ECS consisting of pale white colour somatic embryos have been seen after eight months of culture. Generation of fine embryogenic cells suspension from male flowers turned

yellowish white in colour in *M. Acuminata* cultivars after three "months of culture from embryogenic calli induced from male flowers (Ganapathi *et al*., 2001 and Jalil *et al*., 2003). The cultures have been maintained by sub culturing the cells every ten days till the embryogenic cells were used for germination of somatic" embryos. The yellow pigmentation of cytoplasm of the cells in suspension culture is the indication of embryogenic character of cells in suspension culture of banana cv. Grand nain as well as in cv. Williams (Becker *et al*., 2000).

Zeatin is a natural cytokinin and it promotes embryogenesis due to its enhancing effect on mitotic activity during the early stages "of embryogenesis (Fujimura and Komamine, 1980). The highest percentage (42%) of ECS in cv, Spambia was seen in MS medium supplemented with 0.220 mgL^{-1} of zeatin (Sholi *et al*., 2009). Increasing the concentration of zeatin to 0.440 mgL^{-1} in medium lead to formation of large clumps composed of vacuolated cells without prominent" nuclei, thus reducing their regeneration potentiality (Sholi *et al*., 2009). Similar to these findings we also reported slightly higher rate of ECS establishment i.e., 50% in media supplemented with 0.250 mgL^{-1} of Zeatin.

Table II: Effect of Zeatin during establishment of embryogenic cell suspension of Bhimkol in modified MS (M2) medium

Treatment (Zeatin)	No of replicates	No. of Embryogenic calli used/ replicate	No. of ECS initiated (after 2 months)	Percentage of ECS established successfully
T1 (0.100 mg L^{-1})	5	18	5 ± 0.283	27.78
T2 (0.150 mg L^{-1})	5	19	6 ± 0.179	31.58
T3 (0.200 mg L^{-1})	5	20	8± 0.219	40.00
T4 (0.250 mg L^{-1})	5	20	10 ± 0.501	50.00
T5 (0.300 mg L^{-1})	5	20	9 ± 0.179	45.00

Maturation and Germination of Somatic Embryos

The settled "embryogenic cells from ECS were transferred on sterile filter paper and vacuum dried followed by transferring the filter paper containing cell aggregates on M3 medium supplemented with different concentrations of glutamine viz. 50 mgL^{-1}, 100 mgL^{-1} and 150 mgL^{-1} (Table III). The best result was observed in medium supplemented with" 100 mgL^{-1} of glutamine which gave rise to approximately 2000 numbers of matured somatic embryos per 5mL of settled cell volume (SCV), followed by 150 mgL^{-1} of glutamine which produced approximately 1400 numbers of matured somatic embryos per 5 mL of SCV after 3 months of culture (Figure 1e). Least no. of matured somatic embryos (800) has been observed in M3 medium supplemented with 50 mg L^{-1} of glutamine per 5 mL of SCV after three months of culture. About 2,021 embryos per 5 mL of SCV in banana cultivar 'Da Jiao' (*Musa paradisiac*a, ABB genome) have been obtained on modified SH medium supplemented with 100 mg L^{-1} glutamine (Dai *et al*., 2010).

Amino acids "have been shown to improve somatic embryogenesis in a number of plant species (Loiseau *et al*., 1995). Since glutamine is thermolabile, autoclaving it for 20 minutes at 121°C transforms it into 5-oxo proline (pyroglutamine acid) in the nutritional medium (Sandal *et al*., 2011). Autoclaved glutamine supplementation greatly improved the regeneration of shoots from the callus of maroon *Cucumis anguria* L. and boosted the number of somatic embryos that were produced from hypocotyl and leaf explants" (Jeyakumar *et al*., 2014, Ju *et al*., 2014). The diploid (AA) banana's somatic embryo growth and maturation were induced using autoclaved glutamine (Jalil *et al*., 2003). Using autoclaved glutamine in nutritional medium enhanced the growth of somatic embryos of *Musa acuminata* cv. in contrast to other amino acids like proline. Berangan produced more *in vitro* regenerated shoots from fully developed somatic embryos in a shorter amount of time (Husin *et al*., 2014). Additionally supplementing glutamine in a nutrient medium contributes as a nitrogen source, which helps in improving rate of somatic embryo maturation and subsequent germination of somatic embryos (Morais-Lino *et al*., 2008).

Table III. Effect of Glutamine on the maturation of somatic embryos on modified SH (M3) medium

Treatment (Glutamine)	No. of replicates	Settled cells volume (SCV) of ECS used/replicate	Matured somatic embryos developed/ 5 mL of SCV
T1 (50 mg L^{-1})	5	5 mL	800± 10.126
T2 (100 mg L^{-1})	5	5 mL	2000 ± 14.690
T3 (150 mg L^{-1})	5	5 mL	1400 ± 9.176

Germination of Matured Somatic Embryos

Transferring the developed somatic embryos onto M4 regeneration media supplemented with varying concentrations of BAP- 2 mgL^{-1}, 4 mgL^{-1} and 6 mgL^{-1} of BAP (Table IV). We observed highest percentage (82.80%) of matured somatic embryo germination in M4 medium supplemented with 4 mgL^{-1} BAP. This was followed by 60% of "mature somatic embryos germinated in M4 medium supplemented with 6 mgL^{-1} BAP. The least percentage (36.88%) of germination of mature somatic embryos was observed in M4 medium supplemented with" 2 mgL^{-1} of BAP. These germinated somatic embryos developed green shoot primordia withing thirty days of culture (Figure 1f).

Comparably, MS medium supplemented with 4 mgL^{-1} BAP showed highest percentage (89.53%) of matured somatic embryo germination and subsequently regeneration of shoots in banana cv. Nanjanagud Rasabale (AAB) (Guranna *et al*. 2017). However, the highest percentage (84%) of matured somatic embryo germination and subsequently regeneration of shoots in banana cv. Sabri (AAB) "in MS medium supplemented with 5 mgL^{-1} BAP, while 4 mgL^{-1} BAP declined the germination of somatic embryos to 72% (Khatun *et al*., 2017). On the other hand, highest germination percentage (80%) of matured somatic embryos in" banana cv. Berangan (AAA) at 8 mgL^{-1} of BAP (Darvari *et al*., 2010). Concentration of BAP for *in vitro* germination of matured

somatic embryo and subsequently regeneration to shoots is entirely genotype dependent (Arinaitwe *et al*., 2000). BAP stimulates the cells to increase the uptake of calcium from the nutrient medium leading to increased calcium concentration in the cytosol (Hager *et al*., 1991). Since calcium affects the cytoskeleton, it regulates exocytosis thereby improving the germination of the shoot tips.

Table IV. Effect of BAP concentration on *in vitro* germination of matured somatic embryos of Bhimkol in modified MS (M4) medium

Treatment (BAP)	No. of replicates	No. of somatic cells cultured	No. of somatic embryos germinated	Frequency of Germination (%)
T1 (2 mg L^{-1})	5	250	92 ± 1.165	36.88
T2 (4 mg L^{-1})	5	250	207 ± 0.829	82.80
T3 (6 mg L^{-1})	5	250	150 ± 1.649	60.00

Shoot Proliferation of *in vitro* Regenerated Shoots

Shoot elongation was performed on the *in vitro* regenerated shoots from the germinated somatic embryos in modified MS (M5) media supplemented with varying concentrations of BAP, specifically 2 mgL^{-1}, 4 mgL^{-1} and 6 mgL^{-1} of BAP (Table V). We observed the highest percentage (76%) of shoot elongation "in medium supplemented with 2 mgL^{-1} BAP after 1 month of culture. This was followed by 56% of shoot elongation in medium supplemented with 4 mgL^{-1} BAP. The least shoot elongation percent (32%) was observed in medium supplemented with 6 mgL^{-1} of BAP (Figure 1g). MS medium supplemented with 2 mgL^{-1} BAP" showed highest percentage (70.53%) of shoot proliferation and elongation in banana cv. Nanjanagud Rasabale (AAB) (Guranna *et al*., 2017). After the germination of somatic "embryos, a high concentration of BAP is not essential for further shoot elongation and their proliferation since it leads to reduction in the number of shoots and increases the incidences of abnormality. Using a high concentration of BAP (8 mg L^{-1}) initially induced formation of shoot bud's incidence in banana cv. Berangan however, the high dose of BAP simultaneously increased the formation of abnormal shoots (Jafari *et al*., 2011). Martin *et al*. (2006) and Shirani *et al*. (2009) previously described the negative effects of using high amounts of cytokinin, which cause abnormalities and affect genetic" variability.

Table V. Effect of BAP concentrations for *in vitro* shoot proliferation in modified MS (M5) medium

Treatment (BAP)	No. of replicates	No. of shoot inoculated/ replicate	No. of shoots proliferated after 1 month of culture	Frequency of shoot proliferation (%)
T1 (2 mg L^{-1})	5	50	38 ± 0.219	76.00
T2 (4 mg L^{-1})	5	50	28 ± 0.358	56.00
T3 (6 mg L^{-1})	5	50	16 ± 0.335	32.00

Root Induction and Hardening of *in vitro* Regenerated Shoots

The "*in vitro* regenerated shoots of Bhimkol have been transferred to rooting (R1) medium supplemented with different concentrations of IBA viz. 1 mgL^{-1}, 2 mgL^{-1} and 3 mgL^{-1} (Table VI). In present study, highest percentage (93.33 %) of rooting from *in vitro* regenerated shoots were observed in R1 medium supplemented with" 2 mgL^{-1} of IBA after 1 month of culture (Figure 1h). This was followed by 86.67% of rooting in R1 medium supplemented with 3 mgL^{-1} of IBA. The least percent (56.67%) rooting from *in vitro* regenerated shoots were "observed in R1 medium supplemented with 1 mgL^{-1} of IBA after 1 month of culture. To initiate rhizogenesis, IBA or NAA are most utilised auxins for rooting from *in vitro* regenerated shoots. The maximum rooting has been observed in MS medium supplemented with 2.5 mgL^{-1} of IBA in" *Musa* species (Strosse *et al*., 2006). About 96% of rooting efficiency was observed in plaintain banana cv. Gonja manjaya (AAB) in rooting MS medium supplemented with 1 mgL^{-1} and 2 mgL^{-1} IBA respectively (Tripathi *et al*., 2012, Sinha *et al*., 2018). However, addition of higher concentrations of IBA showed inhibitory effect on rooting. The no. of roots produced per plantlet increased with concentrations up to 1.5 mg L^{-1} IBA in the nutrient medium and declined thereafter with increasing concentration of IBA in banana cv. Sabri (Khatun *et al*., 2017). The root elongation phase was very sensitive to IBA concentration and high concentration (>2.5 mgL^{-1}) of IBA inhibited root elongation, a concentration of 2.5 mgL^{-1} of IBA led to the longest root development in banana cv. Matti (Lohidas and Sujin., 2015).

Table VI. Effect of IBA concentration for rooting from the *in vitro* regenerated shoots in in modified MS (R1) medium

Treatment (IBA)	No. of replicates	No. of *in vitro* regenerated shoots inoculated/ replicate	No. of shoots develop roots after 1 month of culture	Rooting frequency (%)
T1 (1 mg L^{-1})	5	30	17 ± 0.219	56.67
T2 (2 mg L^{-1})	5	30	28 ± 0.219	93.33
T3 (3 mg L^{-1})	5	30	26 ± 0.335	86.67

Acclimatization of *in vitro* Regenerated Bhimkol Plantlets

A total of 395 full-grown plantlets of ~5 cm long with three to four well developed leaves were "washed thoroughly with running tap water to eliminate the trances of agar adhered in roots. These plantlets have been planted in potting mixture of sand: soil: vermicompost (1:1:1) and" transferred to growth chamber in Phytotron unit, AAU Jorhat with 16 hrs light (2500 lux) and 8 hrs dark period at 27±2°C (Figure 1i). These plantlets have been irrigated with ½ strength MS Macro and Micro solutions for two weeks after six to seven weeks upon which 390 plantlets successfully hardened with the survivability rate of 98.73% (Table VII).

The survivability of the transgenic banana plantlets up to 95% when subjected to hardening "in pots filled with soil and farm yard manure in 1:1 ratio" in a containment facility (Tripathi *et al*., 2012). While hardening of transgenic banana in clay pots in a greenhouse by using soil and agropeat in a 1:1 ratio and with a temperature maintained at 26±2°C with 70% humidity had a survivability rate of about 96% (Rustagi *et al*., 2015). The survivability rate of *in vitro* regenerated Bhimkol plantlets have been 98.73%.

Table VII. Survivability rate of *In-vitro* regenerated Bhimkol plantlets.

No of *in vitro* regenerated shoots developed roots after 1 month of culture	No of *in vitro* regenerated Bhimkol plantlets survived after 6 weeks of hardening	Survivability rate (%)
395	390	98.73 %

Survivability rate of *in vitro* regenerated Bhimkol= (No. of *in vitro* regenerated plantlets survived/ Total no. of *in vitro* regenerated shoots developed) x 100 %

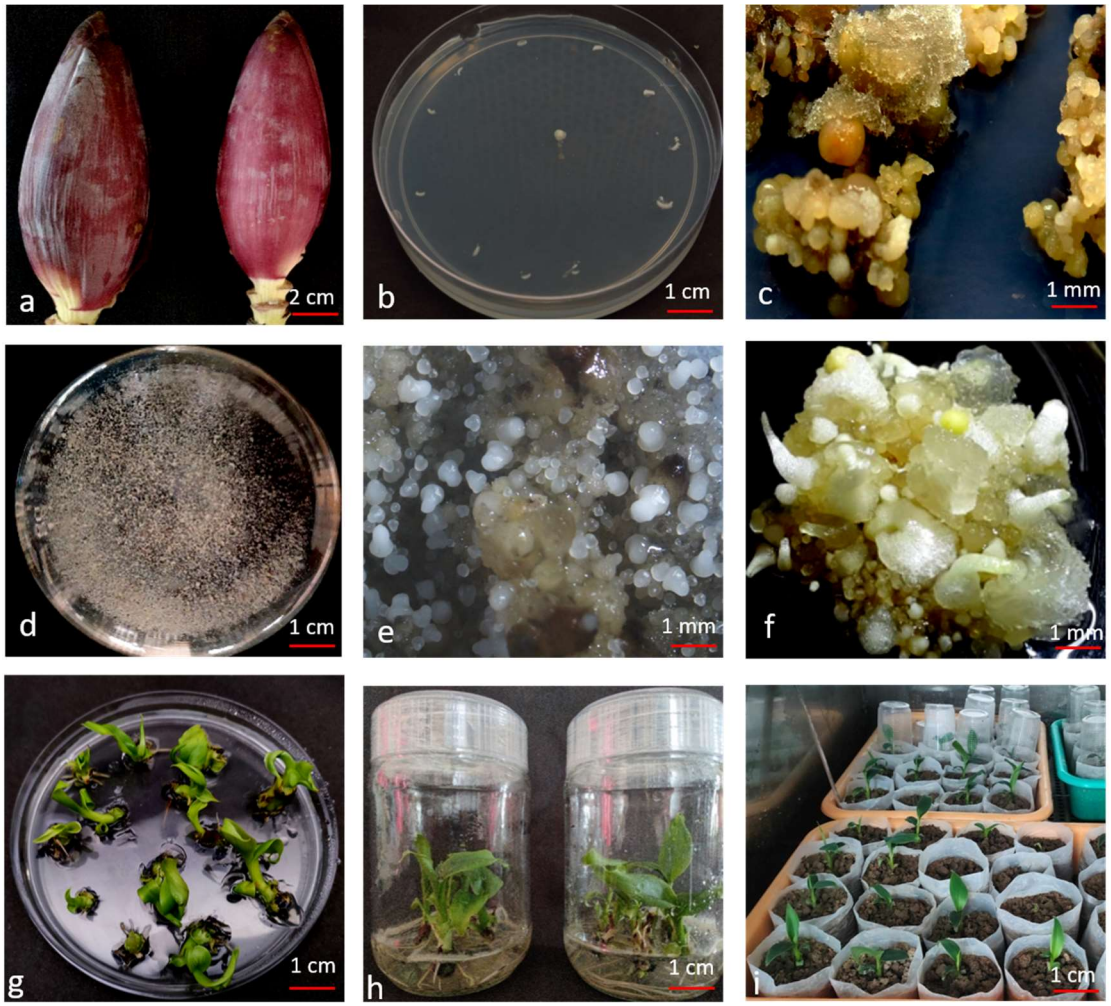

Figure 1

Figure 1: *In vitro* propagation of *Musa balbisiana* cv. Bhimkol utilising ECS developed from the male flowers of Bhimkol. (a) Bhimkol inflorescence; (b) Embryogenic calli initiation from the immature male flowers (c) Embryogenic calli (d) Embryogenic cell suspension (e) Mature ECS cultured for regeneration (f) Shoot regeneration from the mature ECS (g) Shoot proliferation of the regenerated Bhimkol plantlets (h) Regenerated Bhimkol plantlets with well-developed roots (i) *In vitro* regenerated Bhimkol subjected to acclimatization in the phytotron facility at Assam Agricultural University. Jorhat, India.

4. CONCLUSION

We have demonstrated an efficient method of *in vitro* regeneration protocol utilising the ECS developed from the male flowers of Bhimkol. Through the experiment and the results obtained, we may also assume that the response of banana cultivars to different concentrations of plant growth regulators are not the same. This protocol may also serve as a method to develop numerous micro propagules from the ECS which can later be used for genetic improvement research in other diploid cultivars.

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Author Contributions:

Salvinder Singh- Conceptualization, supervision and project administration, Limasunep Longkumer- Investigation, writing, data curation, review and editing, Neha Maske- formal analysis, review and editing, Abhik Gogoi- review and editing

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Data availability:

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations:

Ethics Approval and Consent to Participate:

Not applicable. No new experiments involving human participants or animals were conducted by the authors. The study complies with the institutional, national and international guidelines for the use of cultivated plants in experimental research.

Consent for Publication:

Not applicable.

Competing Interests:

The authors declare no competing interests.

Plant Reproducibility:

This study utilized previously published and publicly available plant data.

Supporting Information:

The supplementary information can be availed from the corresponding author on reasonable request.

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