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RESEARCH ARTICLE

Mass Propagation of Banana (*Musa* sp.) cv. Grand Naine through Direct Organogenesis by Benzyl Adenine Purine and Kinetin

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Abstract

An experiment on *in vitro* banana propagation by meristem cultured from superior quality *Musa* sp. cv. Grand Naine suckers were used as explants in the present investigation. The selected banana explants were free from Cucumber Mosaic Virus (CMV) and Banana Bunchy Top Virus (BBTV) which causes major slack in productivity. Benzyl Adenine Purine (BAP) in five different concentrations (control, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/L) were used for the treatment of shoot proliferation and differentiation and the shoot multiplication rate was maintained by repeated subculturing using same growth regulators. The present study revealed that medium supplemented with BAP produced greater number of shoots (55) and longer shoot (3.0±0.012 cm) on 4.0 mg/L BAP when compared with other treatments. Development of roots were assessed by treating the shoot with five levels of kinetin (control, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) supplemented MS medium. Root development was not observed in the medium devoid of hormone. Whereas, increased number of roots and length of shoots was observed in medium with 1.0 mg/L concentration of kinetin. Survival rate of the plantlets were found to be 84.44% during primary hardening. The plantlets were segregated based on the morphological growth into normal (95.26%), tall (2.1%) and dwarf (2.6%) plants, this condition of abnormality is might be due the somaclonal variations and all the plantlets were subjected to the secondary hardening with garden soil, sand and red soil in the ratio of 1:1:1 in polybags and all the plantlets showed 100% survivability. This tradition of *in vitro* mass propagation method appears to satisfy the increase in demand for disease free and healthy planting materials of banana for commercialization.

Keywords: *Musa* sp. cv. Grand Naine, benzyl adenine purine, kinetin, mass propagation, somaclonal variations.

Introduction

Bananas and Plantains are monocotyledon plants in the genus *Musa* (Family: Musaceae, Order: Zingiberales). They are giant herbs commonly grow up to 3-5 m in height, with no lignification or secondary thickening of stems. The origin of the group is from South to East Asia, occur from India to Polynesia. The plants are distributed mainly on margins of tropical rainforests (Harrison and Schwarzacher, 2007). Banana (*Musa* sp.) is the 4th largest consumed food crop in the world (Vuylsteke, 1989). It is a nutritious fruit rich in carbohydrates and a good source of vitamins. Banana contains low protein levels and can be used to produce large amount of recombinant proteins (i.e. vaccines) (Arvanitoyannis *et al*., 2008). Banana is used as a good source of beverages, fermentable sugars, fragrance, rope, cordage, garlands, shelter, clothing, smoking material, and numerous ceremonial and religious uses (Nelson *et al*., 2006). Banana production has been threatened by a series of abiotic and biotic stresses such as fungi, nematodes, bacterial wilt and viruses, thus, the problems can be avoided or partially controlled through cleaning the plant materials. Therefore, there is a high demand for rapid propagation method for pathogen free plants and safe germplasm exchange (Matsumoto and Silva Neto, 2003). Grand Naine Banana is a high yielding cavendish variety introduced to India from Israel. The plants grow to a height of 5 to 6 ft, the bunches can be harvested within 12-13 months from the date of planting the tissue culture banana plants. Each bunch will be having 10-12 hands with 175-225 numbers of fruits (Nelson *et al*., 2006). Micropropagation has been defined as *in vitro* regeneration of plants from organs, tissues, cells or protoplasts (Beversdorf, 1990) and “the true-to-type propagation of a genotype of explant” (Debergh and Read, 1991). Being parthenocarpic in nature, banana and plantain are propagated vegetatively by means of small shoots or suckers from parent plants. But the rate of multiplication through conventional method is relatively slow and a number of viral diseases (bunchy top virus, banana streak virus) and other diseases also transmitted to next generation and finally the yield becomes very poor which affects the economy of the country. It may be due to the unavailability of the healthy and virus-free suckers.

The banana plant readily produces vegetative suckers next to the mother pseudostem at the base of the plants, with strong vascular connection to the mother. These can be removed from the plant allowing rapid vegetative propagation and multiplication (Harrison and Schwarzacher, 2007). Development of micropropagation techniques has been a major focus of *Musa* research during the past two decades and which have now been well established (Banerjee *et al.,* 1986; Vuylsteke, 1989; Israeli *et al.*, 1995). The relationship existed between the concentration of hormones and the nature of plant explants regenerated. High level of hormonal concentrations resulted in the formation of fewer and abnormal shoots, very difficult to separate. Comparatively, lower levels of hormone gave rise to less number of plantlets. So the optimum hormone concentrations are necessary to rise higher number of plantlets that were tall and normal (Okezie *et al.,* 2000). Several researchers have reported the regeneration of *Musa* sp. through micropropagation (Cronauer and Krikorian, 1986; Krishnamoorthy *et al.,* 2001). Even though many reports are available on *in vitro* propagation, the protocols were complicated. Here, we have attempted a very simple, economical, rapidly multiplying and highly reproducible protocol for large scale micropropagation. Our present work demonstrates plant regeneration from virus free sucker explants of triploid Banana Grand Naine variety and feasibility of recovering and converting to plantlets is also studied.

Materials and methods

Selection of stock explants: Selection of explants is needed to avoid multiplication of pest as well as to take useful variability that many exist in Grand Naine banana plant populations. The desirable genotypes with superior fruit characteristics such as cylindrical bunch, long and thick fingers and better shipment quality must be found in crop field. The Grand Naine were selected from five in a field marked as S1, S2, S3, S4 and S5 nearer to Perandapalli, Hosur, TN, India and stock plants selected for the study were free from disease.

Virus indexing: Virus indexing was done according to Vasane *et al*. (2010). The banana leaves and developing sucker is obtained with the help of sharp knife from the stock explants and the stock explants were sent to certification of virus free status at National Centre For Banana Research, Tiruchirapalli, TN for serological tests and nucleic acid analysis by Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) for the detection of Banana Bunch Top Virus (BBTV) and Cucumber Mosaic Virus (CMV).

Surface sterilization of explants: After the negative results for CMV and BBTV for virus indexing of stock plants, the shoot tip was obtained from developing sucker (about four months of age) of Grand Naine selected stock. The sucker explants were carefully transported to the laboratory with the help of sterile polypropylene bags. In laboratory, the suckers were washed thoroughly under running tap water. The roots and outer leaves were removed until the shoot measured 3.0-3.5 cm in length and 1.0-1.5 cm width at the base. The pale white tissue block (3.0x1.5 cm) containing meristem and rhizomatous base were taken in a beaker. Surface sterilized with 70% ethanol, 0.1% mercuric chloride solution and diluted Tween-20 solution. After that, the explants were rinsed three to four times with sterile distilled water (Bhojwani and Razdan*,* 1983; Razdan*,* 1993; Shiragi *et al.,* 2008).

Inoculation of explants: The surface sterilized meristem tissue blocks were then prepared under stereomicroscope by removal of outer tissue of meristem with the help of sterile scalpel under aseptic condition inside the laminar airflow cabinet until the length reached to 2.0-1.0 cm of its base (Kalimuthu *et al.,* 2007). The individual meristematic dome of tissue was directly inoculated to the tissue culture bottle containing MS medium (Murashige and Skoog, 1962; Shajahan *et al.,* 2011) supplemented with a little concentration of adenine for explants swelling and to attain its maximum size to develop photoactive shoots which is required as per treatment and the bottle were sealed with parafilm.

Incubation: The culture bottles were transferred to the culture room to grow in controlled environment. The temperature of the culture room was maintained with in 25±2^{o}C by an air conditioner (Shiragi *et al.,* 2008). A 16-h light period was maintained with the intensity of 2000 lux for the growth and culture of explants. Relative humidity (RH) was maintained between 40-45%. Meanwhile, during every week interval, the culture room was fumigated with solution of potassium permanganate and formaldehyde.

Maintenance and proliferating of culture stocks (Subculture or transfer): The initial explants subculturing was done and after that, the explants produced well developed shoots from the sucker (Kalimuthu *et al.,* 2007). For subculturing, *in vitro* grown initial explants shoot were cut into small pieces so that each piece would contain about one shoot. Leaf and blackish or browned basal tissues were removed to expose the meristem. Each piece was inoculated into a similar fresh MS medium. It was practiced at the interval of every three weeks. Through series of subculturing, the numbers of *in vitro* shoot stock were increased.

Regeneration of plants from in vitro proliferated buds (Shoot differentiation): In vitro proliferated micro shoots were separated with the help of sterilized scalpel and trimmed the lower base, photoactive shoots into define size and each of the micro shoot was placed on culture medium, supplemented with BAP for shoot differentiation. Five levels of BAP concentration were used as treatment for shoot proliferation in Trial 1 shown in Table 1.

Table 1. Trial 1: MS Agar Media + BAP.

Growth regulator BAP (mg/L)	Number of shoots inoculated	Length of the shoots inoculated in cm
Control	15	2.0
2.0	15	2.0
4.0	15	2.0
6.0	15	2.0
8.0	15	2.0
10.0	15	2.0

Root inductions of regenerated shoots: When the shoots grown about 3-5 cm in length with 2-3 well developed leaves, they were rescued aseptically from the culture vessels and separated from each other and again cultured on freshly prepared medium containing kinetin for root induction (Kalimuthu *et al.,* 2007). For rooting of shoots, activated charcoal in the concentration of 20 mg/L were added and the growth were observed for the height of the shoots, number of shoots, length of the shoots and length of the roots. Five levels of kinetin concentration were used as treatment for root induction in Trial 2 shown in Table 2.

Table 2. Trial 2: MS Agar Media + Kinetin.

Growth regulator Kinetin (mg/L)	Number of shoots inoculated	Length of the shoots inoculated in cm
Control	8	3-4
0.5	8	3-4
1.0	8	3-4
1.5	8	3-4
2.0	8	3-4
2.5	8	3-4

Primary hardening: The primary hardening is carried out for about one and half month in the net pots filled with the coir pith. It takes about 40-50 d to grow according to the height as grade 1-5. To overcome the problem in hardening and acclimatization, the *in vitro* raised plantlets were transferred from bottles to net pots and kept in groups in mist chamber maintained at (80-90%) humidity. The humidity was gradually reduced and plantlets were kept outside the mist house.

Secondary hardening: After 40 d, primary hardened plants with good root ball and shoots were transferred to poly bags which were filled with various potting mixtures such as garden soil, sand and red soil in the ratio of 1:1:1. Then the hardened plants were then transferred to the field.

Results and discussion

Banana plants for virus indexing test: The virus indexations by herbaceous indicator plants are most simple. PCR based techniques and serological technique are needed, though being more complicated, time consuming and requires more lab facilities, thus, the selected banana explants were send to testing laboratory for the detection of Cucumber Mosaic Virus (CMV) and Banana Bunchy Top Virus (BBTV) which causes major loose in banana productivity (Matsumoto and Silva Neto, 2003). Reverse Transcription Polymerase Chain Reaction (RT-PCR) assays were compared for the detection of BBTV which was most sensitive and ELISA immunodetection were inclusive for CMV (Vasane *et al*., 2010). The laboratory results revealed that all the selected five plants as negative. Similar results were reported by Matsumoto and Silva Neto (2003) and Kalimuthu *et al.* (2007).

Establishment of culture explants: When sword suckers were grown on MS medium devoid of any growth regulators no morphogenetic response was observed during initiation of explant. Whereas, sprouting of photoactive green leaves were observed within two weeks of culture on MS medium enriched with adenine. In some cases, within 3 d of incubation explant swelling was observed. The clear appearance of buds was observed after three weeks (Fig. 1). The fresh shoot tips produced two or three shoots in the span of about three weeks incubation when split longitudinally into two halves and subsequently inoculated in the same medium for second initiation (Fig. 2) followed by transferring the four split halves section to semi-solid MS medium containing adenine (30 mg/L) considered as first transfer then second, third and fourth up to eighth transfer (Rao *et al.,* 2009). Upon subculturing, these shoots after 3 weeks interval, each one produced an additional four to six shoots in the subsequent subculture. The average multiplication ratio was even reached to several hundreds to thousands.

Fig. 1. Sprouting of shoot bud from inoculated sucker in MS medium on 22nd d of incubation.

Fig. 2. Shoot development from split longitudinal sucker explants in MS medium after 3 weeks of incubation.

Table 3. Effect of BAP concentration on response of direct organogenesis in MS medium after 3 weeks of incubation.

Conc. of BAP growth regulator (mg/L)	Number of shoots obtained	Length of the shoots obtained in cm.
0.0	15	2.0±0.01
2.0	35	2.3±0.01
4.0	55	3.0±0.012
6.0	40	2.8±0.011
8.0	42	2.4±0.01
10.0	25	2.5±0.02

Fig. 3. Induction of multiple shoots of Banana cv. Grand Naine produced on MS medium containing BAP.

A-Explants during inoculation in BAP containing medium

B-Multiple shoots with 4.0 mg/L BAP at 3 weeks after inoculation

In vitro proliferation of shoot buds: In BAP enriched medium, the shoot buds soon elongated into slender shoots with well developed branching and leaves from the inoculated sucker. MS medium augmented with benzyl adenine purine showed comparatively less response. MS Medium enriched with 4.0 mg/L showed maximum response as compared to other BAP concentrations (control, 2.0-10.0 mg/L) assessed. BAP at 4.0 mg/L showed a maximum of 55±0.21 from 15 numbers of shoots and the shoot length was maximum (3.0±0.012 cm). The results were shown in Table 3 and Fig. 3. BAP an adenine type cytokinin has been reported to be the best cytokinin for multiple shoot proliferation for Rutaceae and other members (Nayak *et al.,* 2007; Bohidar *et al*., 2008).

The concentration of cytokinin used significantly affected the percentage of shoot regeneration, shoot numbers and shoot length. Cytokinin concentration has been several times reported to be decisive for shoot proliferation and elongation of many medicinal plant species (Rout and Jain, 2004). Similar results of BAP (2.5 mg/L) stimulate shoot elongation and rooting in medium after three weeks of inoculation (Matsumoto and Silva neto, 2003) were obtained, comparable report also given by Strosse *et al.* (1991). Similar results were reported in case of *Agele marmelos* (Nayak *et al.,* 2007) and in Rutaceae (Bohidar *et al*., 2008).

In vitro root generation from the developed shoots: Production of plantlets with profuse rooting *in vitro* is important for the successful establishment of regenerated plants in soil. In case of kinetin, shoot proliferation and roots can be initiated on the same medium employed for multiplication or on media with lower ionic concentration. Shoots are elongated and rooted spontaneously after three weeks of incubation in culture on the MS medium containing 0.5-2.5 mg/L of kinetin. MS medium augmented with kinetin showed less response. MS medium with 1.0 mg/L shows maximum response for both shoot proliferation and root formation when compared to the other concentrations of kinetin (Table 4). Kinetin at 1.0 mg/L showed maximum of 12.0±0.06 numbers of shoots from 8 inoculated shoots. The average length of the shoot was maximum (6.0±0.029 cm) and after 3 weeks of incubation, root formed were of 6.5±0.032 cm in length with maximum of 4.0±0.02 roots obtained in the same medium. The results obtained were shown in Table 4 and Fig. 4. The stimulatory effect of kinetin rooting was reported by Matsumoto and Silva Neto (2003) in other *Musa* sp.

Fig. 4. Root formation of Banana cv. Grand Naine on MS medium containing kinetin.

Table 4. Effect of kinetin conc. on response shoots and root proliferation in MS medium after 3 weeks of incubation.

Conc. of kinetin growth hormone (mg/L)	No. of shoots obtained	No. of roots per plantlet	Length of shoots obtained in cm	Length of roots in cm
0.0	8.0±0.012	0.0	4.0	0.0
0.5	9.5±0.04	2.0±0.01	4.5±0.025	4.7±0.021
1.0	12.0±0.06	4.0±0.02	6.0±0.029	6.5±0.032
1.5	11.5±0.06	2.0±0.01	4.9±0.024	5.0±0.026
2.0	10.3±0.05	1.0±0.01	4.3±0.02	4.0±0.018
2.5	9.7±0.07	1.0±0.01	3.9±0.022	4.1±0.021

Table 5. Morphological profile of Banana cv. Grand Naine plantlets upon acclimatization.

Properties	Normal	Tall	Dwarf
Occurrence	181	4	5
Height (cm)	10.0	15.5	5.0
Leaf arrangement	Alternate	Alternate	Opposite
Leaf colour	Green	Green	White patches
Leaf morphology	Narrow, pointed end	Broad	Poorly developed

Fig. 5. Well established meristem derived banana plants in poly bags during secondary hardening.

Acclimatization: Plantlets for primary and secondary hardening: The plantlets were rigorously inspected and ensured free from disease were subjected to primary and secondary hardening. Segregation of these plants into normal, tall and dwarf was carried out. A total of 225 plantlets were subjected to primary and secondary hardening out of which 190 plantlets survived. The survival rate of plantlets was about 84.44% in coir pith net pots. Similar results were reported by Jasari *et al.* (1999) and Shiragi *et al.* (2008) who showed 83.3% and 92% respectively.

Normal plants: The normal plants were in good health, green, with alternate leaf arrangement, broad leaf area, roots entangled in the growth medium and formed a root ball from young roots. The normal plantlets growth was 95.26% during hardening and results were given in Table 5 (Fig. 5). Similar percentages (98.8%) were obtained by Ramage *et al*. (2004) and Vasane *et al*. (2010).

Tall plants: Tall plants were approximately 2-3 times taller than the normal plants, tapering and with pointed leaves; internodes distance more as compared to the normal ones. Four plants were obtained as taller one and the percentage of occurrence is 2.1%. However, 0.07% of tall plants were obtained by Vasane *et al*. (2010).

Dwarf plants: Dwarf plants were most difficult to identify during primary hardening, these dwarf plants were identified mostly towards the end of secondary hardening and remain stunted even after providing either optimum growth conditions for a longer period. Five plantlets remained stunt and don't show any growth. The occurrences of dwarf plants while hardening were 2.6%, however, the result obtained by Vasane *et al*. (2010) was only 0.23%.

Conclusion

The present study concluded that the MS medium supplemented with 4.2 mg/L BAP showed maximum response for shoot proliferation whereas, 1.0 mg/L kinetin provoke greater number of root formation and differentiation. Hormonal concentration mentioned in this study can be recommended for mass propagation of *Musa* sp. cv Grand Naine. The study may be extended for different concentrations of other growth hormones.

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