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Optimization of *In Vitro* Micropropagation Protocol for Banana (*Musa Sapientum* L.) Under Different Hormonal Concentrations and Growth Media

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***Abstract* – This study was aimed to develop a simple, comprehensive and efficiently repetitive protocol for micropropagation of banana (*Musa sapientum* L.) using shoot meristem. After growing in-vitro cultures on different hormonal combinations, Murashige and Skoog's medium supplemented with BAP and IAA (5.0 + 1.0 mg/l, respectively) and 10% CW were found to be most efficient and productive combination for shoot proliferation. Antibiotic (cefotaxime) was used to check the endogenously born bacterial contamination. Initiation of shoot bud and establishment of culture from shoot meristem was achieved on liquid media. Further shoot proliferation of cultures up to 3 batches of 15 days each was achieved on the agarose gel solidified hormonal supplemented media after culture establishment. The proliferated shoots were excised and transferred to different root induction media, which resultantly showed that MS media supplemented with IAA (2mg/l), was the most efficient root inducing media. Rooted plantlets after primary and secondary hardening were transferred to the green house. Finally, these disease free plants were successfully established in soil.**

***Keywords* – Micropropagation, Regeneration, *Musa Sapientum* L, Shoot Meristem.**

I. Introduction

Edible bananas (Musa spp.) are the major staple food for rural and urban consumers in the tropical and subtropical countries and an important source of rural income. The genus Musa (family *Musaceae*) originates in Asia (Simmonds, 1962). Cultivated banana is derived from two diploid species of genus *Musa*. *M. acuminate* (Malaysia) and *M. balbiciana* (India) parent genomes (Stover & Simmonds, 1987; Simmonds, 1962; George *et al.,* 2000). Banana is a good source of carbohydrates, proteins, vitamins and minerals. Many pest and diseases (especially viral diseases i.e. banana mosaic virus) constrain banana production which resulted in serious consequences for environment through the application of pesticides. Thus, major constraints in the banana production system are the non-availability of disease-free, true-to-type planting material, low fertility due to triploidy, slow propagation and long time span from one generation to the next generation. Classical breeding is difficult Because of its high degree of sterility and polyploidy of the edible varieties (Stover & Simmonds, 1987). Bananas belong to group of crops which are normally propagated through vegetative parts of the plant because almost all cultivated banana cultivars are triploid, seedless, or seed sterile. The materials used for conventional propagation include corms, large and small suckers, and sword suckers (Cronauer & Krikorian, 1984; Arias, 1992; Haq and Dahot, 2007).

Mass propagation of selected genotypes, somaclonal variation techniques, genetic engineering and other biotechnological applications can be utilized for banana crop improvement which is based on reliable plant regeneration protocols. Tissue culture also plays a vital role in the distribution of germplasm, conservation, safe exchange of internal planting material and rapid propagation of newly selected hybrid cultivars. Several researchers have reported the regeneration of Musa spp. via micro propagation (Cronauer & Krikorian, 1986; Jarret, 1986; Diniz *et al.,* 1999; Nauyen & Kozai, 2001; Krishnamoorthy *et al.,* 2001; Kagera *et al.,*., 2004; Muhammad *et al*, 2004; Roels *et al.,* 2005; Madhulatha *et al.,* 2004). But, propagation percentage and repeatability of the method are matters of concern which ultimately need a comprehensive, repeatable and applied method for a wide range of genotypes to facilitate disease free production of banana crop on commercial scale. For in vitro micropropagation of banana, bacterial contamination is a big problem. Although initially surface sterilization works, latter on microbial contamination at the base of the explant appears within 7 to 15 days after inoculation. Huge number of explants is destroyed in the culture due to endogenous bacteria (Habiba *et al.,* 2002).

The present study suggests a rapid banana multiplication protocol from shoot meristem by using a medium with optimized concentration of auxins/cytokinins. Many reports are available on in vitro propagation with complicated protocols but less shoot proliferation percentage which eventually yield less number of regenerated plants per culture. Here, we reported a very simple, efficient, economical, rapidly multiplying and highly reproducible protocol for the micro-propagation of banana on commercial scale.

II. Materials and Methods

Selection of plant material: Suckers of selected plants from elite plants of high yielding Chinese variety 8818-Williams were collected and meristem was selected as explant. It was washed in running tap water for 15 - 20 min. The ensheathing leaf bases were removed from the lower stem leaving the young leaves around the meristem.

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The explants material was rinsed in 70% ethanol for I minute, followed by 15% sodium hypochlorite solution for 10 min.

Immersion of explants in antibiotic: The surface sterilized explants were immersed in screened antibiotic cefotaxime (200 mg/l) for one hour to ensure contamination free cultures.

Treatment allocation: After washing thrice in sterile distilled water, the explants were inoculated aseptically in MS Medium (Murashige & Skoog, 1962) containing 30-g/l sucrose for culture establishment. The MS medium was variously supplemented with 6-benzyl amino purines (BAP), and Indole acetic acid (IAA) in various combinations as shown in Table I. After the interval of 14 days, the established shoots were sub cultured to liquid media and incubated on electric shaker at speed 30 rpm. Established cultures were then routinely transferred every 2 weeks by subdividing shoot clusters to the solid media. Average length of shoots was calculated after every sub culturing. For shoot proliferation on solid media, MS media supplemented with various combinations of Bap, IAA and coconut water was used (Table II).

Root development and shifting to field: Four different auxins (ABA, NAA, IBA and IAA) with varying concentrations were employed to check their effect on root induction. Each auxins was applied in four different concentration (0, 0.5, 1.0 and 2.0 mg/l).For root induction, the well grown shoots were separated and transferred to MS media containing varying concentrations of auxins as described in Fig. 3. The pH of the medium was adjusted to 5.8 before autoclaving and temperature was maintained at 26 ± 2 °C and 16 h photoperiod (4000 lux) (Dooley, 1991). The *in-vitro* raised plants were transferred from bottles to net pots and kept in mist chamber maintained at (80 – 90%) humidity. The humidity was gradually reduced and plantlets were kept outside the mist house. After 20 days, they were transferred to polythene bags which were filled with peat moss containing farm yard manure (FYM), sand and red soil in the ratio of 1:1:1. The hardened plants were then transferred to the field.

III. Results & Discussion

The meristematic shoot tip explants were inoculated on MS medium with eight different combinations of BAP and IAA. Among the various treatments, the effective results were obtained from these eight combinations given in the Table 1. After 2 weeks, explants got swelled changed colour to green and produced shoots lets. The best medium for culture establishment was MS + 5.0 mg/l BAP + 1.0 mg/l IAA and average time required was 15 - 21 days .The result showed that the best shoot proliferation was in BAP + IAA (5.0 + 1.0 mg/l), followed by the next combination of BAP + IAA (4.0 + 1.0 mg /l). The poor response of shoot initiation was observed both in first (1.0+ 1.0 mg/l of BAP + IAA) and last (7.0 + 0.25 mg/l BAP + IAA) combinations of the growth regulators. The results showed that 90% contamination free cultures were obtained by soaking the explants in 200 mg/l Cefotaxime for one hr. When these antibiotic treated explants were cultured in MS, they produced healthy shoots *Fig. 1*.

Sub-culturing of the initially established shoots was done to the liquid media with same hormonal supplements which induced multiple shoots. After five sub-culturing, the clump formation occurs and the capability of culture to further divide gradually becomes low. The proliferating axillary buds were well defined, pale green and 2.0-4.0 cm long with bulbous base and thin pointed tips. After 4-5 weeks, threefold increase in multiplication was seen. At every subculture in the same media resulted in three to four fold increases in multiplicated axillary buds.

The explants of 10-12 weeks in MS with BAP (5.0 mg/l) and IAA (1.0 mg/l) medium attained the stage of vigorous proliferation, were divided into smaller clumps. Each clump consisting of 5-6 shoots was transferred to the shoot proliferation solid medium (Table 2). Results clearly showed that 5.0 BAP+1.0 IAA+10%CW media proved to be most productive for shoot proliferation. When these shoots were sub-cultured to the same media, gave rise to a three to four fold increase in proliferating clumps and 3- 4 elongated shoots with root initiation. The elongated shoots were excised and cultured separately in same fresh medium to encourage formation of long shoots broad leaves and basal roots.

The highest number of shoots were produced on MS supplemented with 5.0 mg/l BAP +1.5 mg/l IAA + 10 % CW and 6.0 mg/l BAP + 1.5 mg/l IAA +10% coconut water (Table 2-*Fig. 2*). Proliferating shoots continue to produce axillary buds on the same fresh media while elongated shoots were transferred to root induction media for root development.

Four different auxins (IAA, NAA, IBA and ABA) were used in four different combinations (0mg/l, 0.5mg/l, 1.0mg/l and 2.0mg/l) in MS supplemented medium for root induction. The results showed that MS medium supplemented with IAA (2.0mg/l) gave best results which produced 60% root induction *Fig.3*.

In the present investigation, shoot meristems were cultured on agar (semisolid) medium as well as on liquid medium for shoot initiation in contrast to Kalimuthu *et al.,* (2007) where they used only semisolid media. In liquid media, the shoot buds showed vigorous multiplication in the form of clumps. The multiplying shoot tips on liquid media were incubated at electric shaker (30rpm) in order to enhance aeration in the media. Better results were shown in liquid media at shaker. After culture establishment, shoot lets were transferred to agarose based solid medium for shoot proliferation. This multiple shoot formation by meristem slices on agar medium confirms the report by Mante & Tepper (1983) and Kalimuthu *et al.,* (2007). Among eight different combinations with various concentrations of BAP and IAA were used in this study to analyze the shoot initiation and shoot multiplication and resultantly MS medium of 5.0 mg/l of BAP and 1.0 mg/l of IAA+10% coconut water showed good results both for shoot initiation and multiplication.

The findings of present study showed that the shoot sub-culturing in the same hormonal supplemented medium gave rise to a three to four fold increase in shoot

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proliferation which resultantly developed healthy rooted plants from each clump in root induction media. The *in-vitro* raised plantlets were successfully established in the polythene pots containing FYM+ clay+ garden soil in 1:1:1 ratio. Successful rooting was attained within 35-40 days while Vessey & Rivera (1981) reported root formation occurred 50 days after shoot development. Berg and Bustamante (1974) noted that it needed 2-3 months for root formation.

In present study, we formulated very simple protocol using only one hormonal combination i.e. MS with 5.0 mg/l of BAP and 1.0 mg/l of IAA+10% coconut water for culture initiation, shoot multiplication and elongation. Only one hormonal combination was optimized for successful root induction in banana i.e. MS supplemented with 2mg/l IAA. Where in contrast (Arias,O., 1992,Cronauer and Krikorian, 1986; Jarret, 1986; Diniz *et al.,*., 1999; Nauyen and Kozai, 2001; Krishnamoorthy *et al.,*., 2001; Kagera *et al.,*., 2004; Gubbuk and Pekmezc, 2004) used media of different kinds at each step. The protocol optimized in the present study could be used for the large scale *in-vitro* production of the plantlets of the banana.

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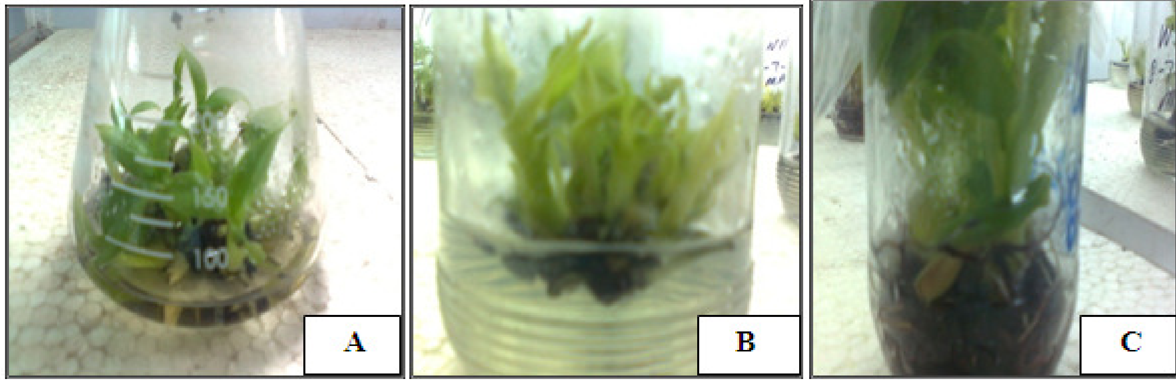

Fig.1. Different developmental stages of banana growth through shoot meristem mediated direct regeneration (A) Growing clumps from shoot tip in liquid media (B) Multiplying clumps at solid media (C) Plantlets exhibiting roots

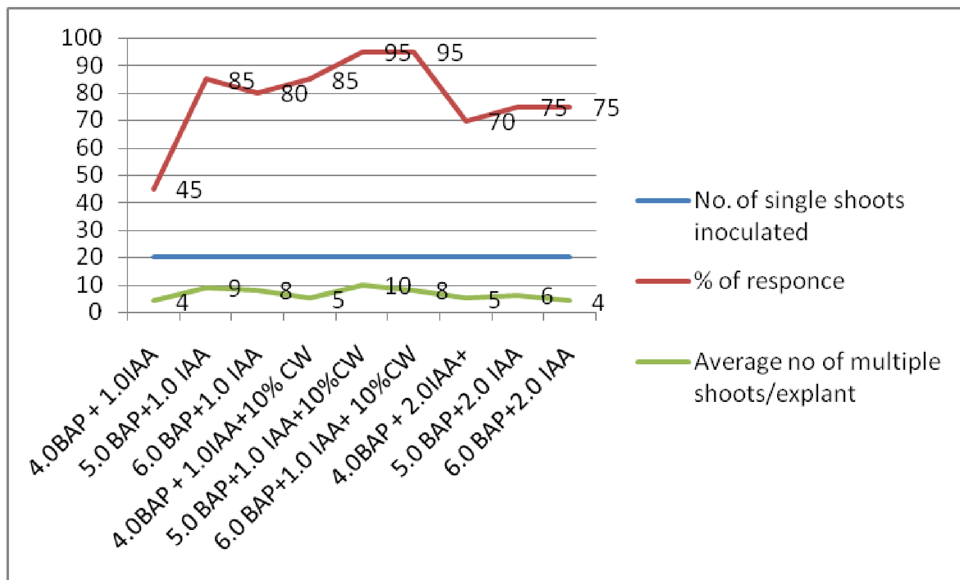

Fig.2. Effects of different combinations of auxins, cytokinins and coconut water (CW) on the production of multiple shoots and roots in MS medium

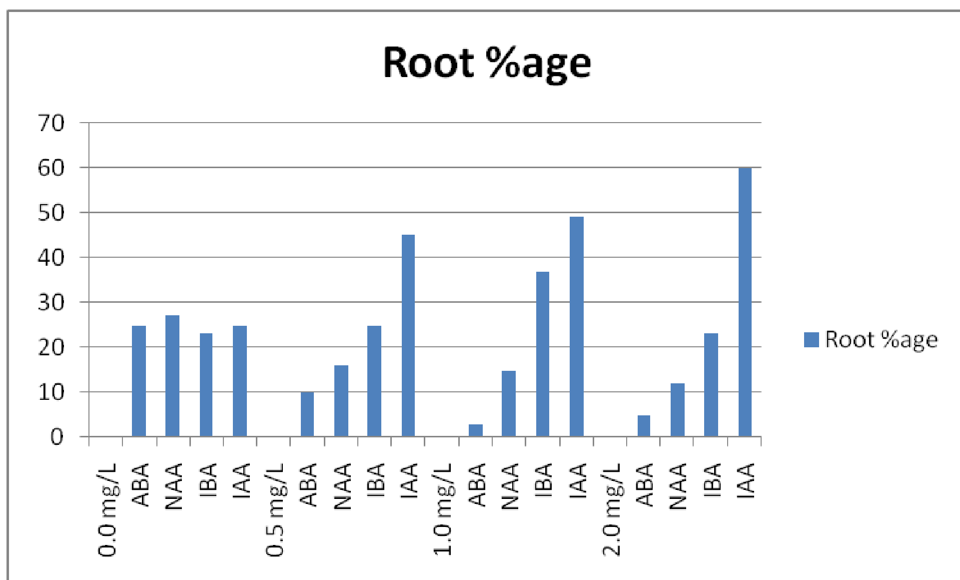

Fig.3. Effects of different concentrations of auxins (ABA, NAA, IBA and IAA) on root induction in banana

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Table I: Effect of different concentrations of plant growth regulator BAP and IAA on in vitro shoot proliferation from shoot tip explants of banana

Concentration of BAP + IAA In MS Medium (Mg/L)	No. of Explants Inoculated	Percentage (%) Of Explants Forming Shoots	No. of Shoots Per Explant	Average Time Required (Days)	Average Length of Shoots (cm)
1.0 + 1.0	25	50	1	20-25	2.5
2.0+1.0	25	58	2	20-25	2.5
3.0+1.0	25	77	3	18-24	2.5
4.0+1.0	25	89	2	18-24	3.0
5.0+1.0	25	90	4	15-21	4.0
6.0+1.0	25	76	2	15-21	4.0
7.0+1.0	25	71	1	16-22	3.5
BASAL MEDIUM	25	50	1	25-	2.0

Table II: Effects of different combinations of auxins, cytokinins and coconut water (CW) on the Production of multiple shoots and Roots in MS medium

Hormonal supplements (mg/L)	No. of single shoots inoculated	No. of single shoots showed multiplication	% of response	Average no of multiple shoots/explant	Average time required (days)	Root development
4.0BAP + 1.0IAA	20	9±2.0	45	4±0.8	35-40	-
5.0 BAP+1.0 IAA	20	17±1.0	85	9±1.2	35-40	-
6.0 BAP+1.0 IAA	20	16±1.0	80	8±0.9	35-40	-
4.0BAP + 1.0IAA + 10% CW	20	17±2.5	85	5±1.5	35-40	-
5.0 BAP+1.0 IAA + 10%CW	20	19±1.8	95	10±1.4	35-40	-
6.0 BAP+1.0 IAA + 10%CW	20	19±1.1	95	8±2.0	35-40	-
4.0BAP + 2.0IAA	20	14±1.0	70	5±1.1	35-40	Rudimentary roots develop
5.0 BAP+2.0 IAA	20	15±1.6	75	6±0.9	35-40	Rudimentary roots develop
6.0 BAP+2.0 IAA	20	15±0.7	75	4±1.0	35-40	Rudimentary roots develop

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