

Original Research Article

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Establishment of Efficient *In-vitro* Regeneration Protocol in ‘Malbhog’ Banana (*Musa paradisiaca*) using MWCNTs and Plant Growth Regulators

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ABSTRACT

Keywords

Multi walled carbon nano tubes, Functionalized carbon nano tubes, *In-vitro* regeneration of Malbhog banana

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The present study was performed to establish an efficient and standard protocol for in vitro propagation of ‘Malbhog’ (*Musa paradisiaca*, AAB genome) banana under suitable culture conditions and to study the effect of growth hormones BAP & NAA on the growth of explants. Further, for the first time we report here the effect of MWCNTs (Multi walled carbon nano tubes) like FCNTs (Functionalized carbon nano tubes), D1 and D2 in ‘Malbhog’ *In-vitro* regeneration. It was also observed that TDZ at 0.11 mg/L showed the highest response (7.5 shoots per explants) in terms of shoot proliferation where as root induction was found to be highest in the MS media supplemented with 1 mg/L NAA. Also, the explants grown in MS Media with FCNTs at 100 µg/mL (8.5 shoots per explants) exhibited acceleration of shoot proliferation in comparison to control without FCNTs.

Introduction

‘Malbhog’ (*Musa paradisiaca*, AAB genome), is a banana cultivar which has high commercial value in the North Eastern part of India because of its nutritional value, sweet taste and aroma. As the quality planting materials are always in demand so this requires a fast and efficient mass production of banana saplings for commercial cultivation. Tissue culture raised ‘Malbhog’

banana plants will reduce the problem of Panama wilt, as disease free quality propagules would be available for planting (Thangavelu *et al.*, 2012). Micropropagated banana plants produce bunch with higher weight, more fingers and hands and more uniform fruit size and shape (Tomekpe, *et al.*, 2011). Various methods have been developed earlier for rapid regeneration and to overcome the shortage of planting materials (Barker, 1959; De Smet *et al.*, 1995). Methods have

been developed for 'Malbhog' micropropagation with the effect of different plant growth regulators (Sugandh Suman and Harsh Kumar, 2015; Goswami and Handique, 2013; Roy *et al.*, 2010). Further, in very recent times, nano particles have also been used in tissue culture for better regeneration efficiency, control of contamination and other benefits (McGehee, Diamond, *et al.*, 2017; Helaly, Mohamed Naser *et al.*, 2014). Silver nano particles have been employed to evaluate the effect on banana growth and development. Nano silver have been found efficient in controlling contaminations and increase in multiplication of shoots by three fold higher than in the control experiments (Dang Giap *et al.*, 2018). The growth of tobacco cell culture (callus) which can be affected and highly enhanced by the introduction of multiwalled carbon nanotubes in the growth medium (Khodakovskaya *et al.*, 2012). The effect of CNTs seems to increase in the growth of germinated embryos and root number (Rania A. Taha, Mona M. Hassan, *et al.*, 2016).

Since there are report on use of carbon nano tubes in tissue culture for better regeneration and development in other crops, therefore, multiwalled carbon nano tube can be used to develop efficient regeneration protocol for 'Malbhog' banana. However, there is no report available on use of multiwalled carbon nano tubes in regeneration of 'Malbhog' cultivar of banana. In the present study, we report here for the first time the effect of multi walled carbon nano tubes in successful regeneration of 'Malbhog' banana.

Materials and Methods

Plant materials

Musa paradisiacal 'Malbhog' (AAB) suckers was collected from farmyard of Sivasagar district, and Chroma hydroponic nursery,

Dibrugarh, Assam.

Sterilization

The suckers were excised to a size about 6cm and kept in Tween-20 solution for 30 minutes followed by washing under running tap water for 20 minutes. The suckers are again excised to a size about 3cm by removing two layers and then dipped in a solution of 1% bavistin (Hi-Media®), 1% chloramphenicol (Hi-Media®) and 2% sodium hypochlorite (Hi-Media®) for 45 minutes as reported by Goswami and Handique (2013). The treated explants were then rinsed 3 times in sterile distilled water. The explants were then dipped in 50mg/L – 100mg/L ascorbic acid solution for 1 hour, followed by 3-4 rinses in distilled water. The final step of sterilizing the explants was done by removing one scale and treating them with 0.1% mercuric chloride solution, followed by 3 rinses in sterile distilled water.

Plant regeneration

Suckers of about 2-3 cm were inoculated in the culture bottle. The solid culture medium contained MS macro and micro nutrients (Murashige and Skoog, 1962), 30g/L sucrose (Hi-Media®) and 8g/L agar (Hi-Media®). The different concentrations of plant growth regulators were tested: BAP (2.5-7.5 mg/L), TDZ (0.05-0.22 mg/L), and in combination of BAP with NAA (0.5-2 mg/L); TDZ with NAA (0.5-2 mg/L) respectively. The explants were also inoculated in medium supplemented with different concentrations of FCNT (Functionalised carbon nano tubes), D1 and D2 at 10, 50, 100 µg/mL respectively. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were kept at 25 ± 2°C with 16 hours illuminations at 2000 lux and 8 hours dark phases and the observation was recorded after 7days of incubation. The regenerated explants were subcultured for 3-4

times on the same media at an interval of six weeks to get multiple shoots. The well grown elongated shoots were transferred to half strength MS medium containing 0.5 and 1.0mg/L NAA for the induction of roots and also to MS medium without hormones. The well rooted plants were then removed from media and washed off for agar traces. The plantlets were transferred to polybags in greenhouse and then to fields.

Results and Discussion

Establishment of aseptic culture

Establishment of aseptic culture was difficult, because the explants were taken from sucker in field conditions. Further, the browning of explants is reduced by treating the explants with ascorbic acid (100mg/L) at different exposure time (30-60 min). Treatment with ascorbic acid for 60 minutes was found effective in culture establishment.

Induction of shoots

The explants were cultured on MS media supplemented with growth hormone alone or in combinations. The different concentrations of BAP (2.5, 5.0, 7.5mg/L), TDZ (0.05, 0.11, 0.22 mg/L) and NAA (0.5, 1.0, 1.5 mg/L) were used alone and in combinations. BAP at 2.5 mg/L showed better result as 7.25 shoots per explants was produced over all the other treatments (Table 1). The lowest rate of initiation of shoot was observed when BAP concentration was increased to 7.5mg/L.

When TDZ was used alone for shoot initiation it was observed that TDZ at 0.11mg/L produced maximum number of shoots (7.5 shoots per explant) as shown in (Table 1), over all the other treatments of TDZ. The effect of BAP and TDZ with combination of NAA was studied for shoot multiplication and it was observed that BAP at 2.5mg/L + 1mg/L

NAA (7.4 shoots per explants) resulted a maximum number of shoots. Similarly, TDZ at 0.11mg/L + 1mg/L NAA (6.3 shoots per explants) showed better result.

Shoot induction on MS with Multi Walled Carbon Nano tubes (MWCNT's)

A separate study was carried out to observe the effect of multi walled carbon nano tubes along with BAP on shoot initiation and multiplication. Functionalised carbon nano tube (FCNT), D1 and D2 were used in culture media after dispersing the nano tubes in Ultrasonicator (Fig. 1D). FCNT, D1 and D2 were studied at 10, 50 and 100µg/mL for shoot induction. MS media having 2.5mg/L BAP with 100µg/mL FCNT (Fig. 1G) produced maximum of 12.5 shoots per explants as shown in (table 2), compared to treatments without the effect of FCNTs. Similarly, when D1 is used in media at 2.5mg/L BAP + 50µg/mL D1 showed better result over all the other treatments of D1. BAP at 2.5mg/L + 100µg/mL D2 produced maximum number of shoots. Therefore the use of FCNTs in the growth medium showed positive response for efficient regeneration with highest number of shoots compared to all the other treatments (Table 2).

Rooting of the proliferated shoots

Different concentrations of NAA (0.5 mg/L and 1.0mg/L) were supplemented on both, full and half strength MS basal medium. The NAA concentration at 1.0 mg/L showed positive response in root initiation over all the other (Fig. 1I). Full strength MS medium containing NAA showed very poor response in root initiation. The roots were also developed when MS medium was not fortified with hormones.

The results of the study reported here showed that the use of ascorbic acid for 60 minutes

was efficient in establishment of culture otherwise; it would lead to death of explants due to browning. Similar results were reported by Goswami and Handique (2013), when they treated the explants with ascorbic acid, citric acid and a solution of ascorbic acid

and citric acid 100mg/L for 1 hour before surface sterilization and 50 mg/L Ascorbic Acid, 50 mg/L Citric Acid and a solution of Ascorbic acid and citric acid 100mg/L for 10minutes.

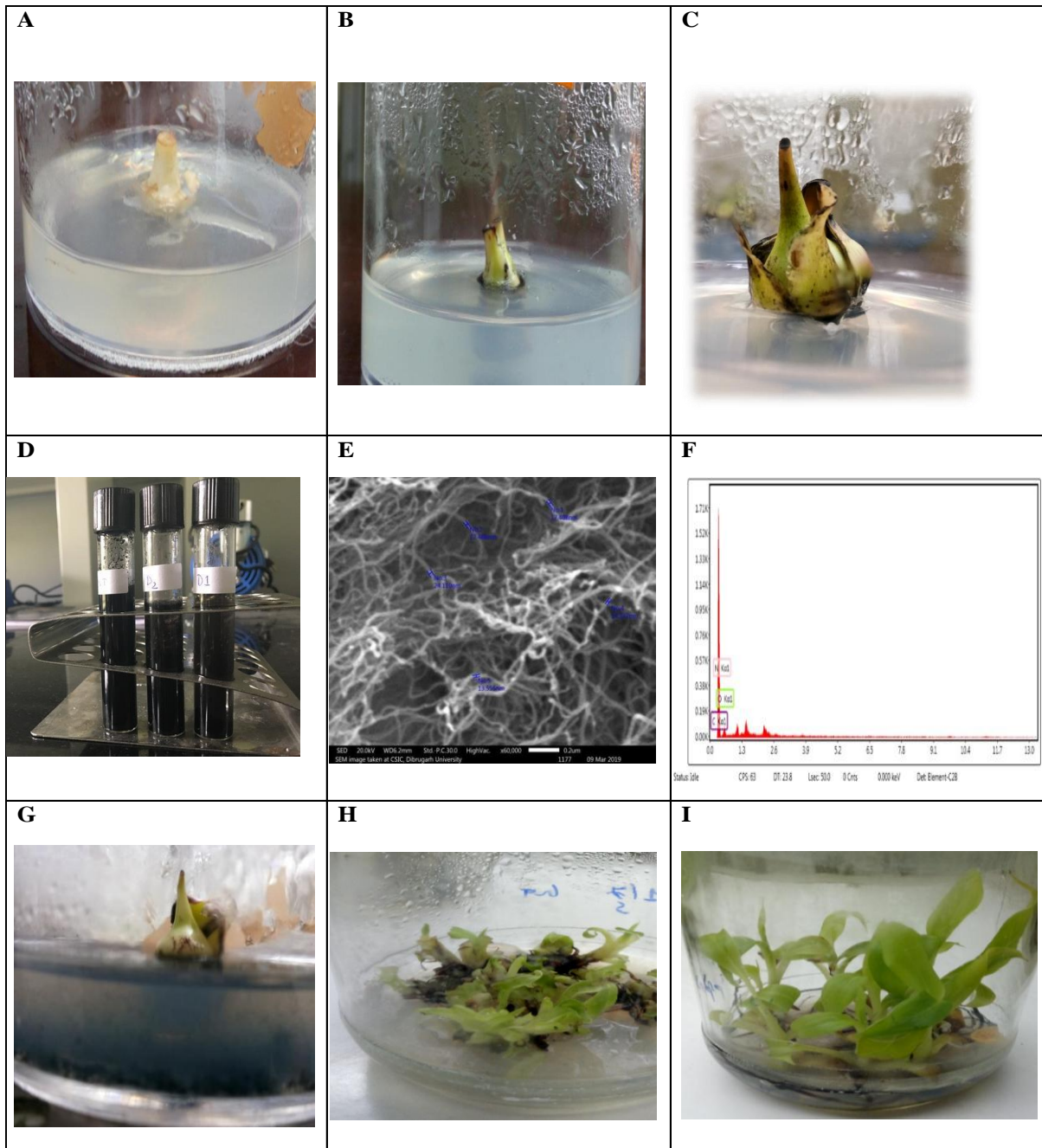
Table.1 MS basal medium treatments with different concentration of growth regulators for shoot multiplication

Treatments	Plant growth regulators (mg/L)				No.of shoots per explant	Shoot length (cm)
	BAP mg/L	TDZ mg/L	BAP + NAA mg/L	TDZ + NAA mg/L		
T1	-	-	-	-	0.6	0.55
T2	2.5	-	-	-	7.25	4.80
T3	5	-	-	-	3.5	3.60
T4	7.5	-	-	-	1.75	2.63
T5	-	0.05	-	-	2	3.10
T6	-	0.11	-	-	7.5	4.80
T7	-	0.22	-	-	1.5	3.60
T8	-	-	1 + 0.5	-	0.8	2.25
T9	-	-	2.5 + 1	-	7.4	4.36
T10	-	-	5 + 2	-	4.2	2.23
T11	-	-	-	0.05 + 0.5	1.5	3.36
T12	-	-	-	0.11 + 1	6.3	3.96
T13	-	-	-	0.22 + 2	1.2	2.50

Table.2 MS medium supplemented with MWCNT's and optimum hormone concentration

Treatments	MWCNT's	BAP (mg/mL)	No. of shoots per explant	Shoot length (cm)
	FCNT (µg/mL)			
T1	10	2.5	7.23	4.6
T2	50	2.5	7.26	4.8
T3	100	2.5	12.5	5.2
	D1 (µg/mL)			
T4	10	2.5	4.5	3.26
T5	50	2.5	7.0	4.5
T6	100	2.5	4.5	3.1
	D2 (µg/ml)			
T7	10	2.5	4.0	2.86
T8	50	2.5	3.2	2.7
T9	100	2.5	7.85	4.8

Fig.1 Regeneration of *Musa paradisiaca* ‘Malbhog banana’ through different concentration of plant growth regulators and multi walled carbon nano tubes. (A) Inoculation of meristematic shoot tips. (B) Establishment of culture (green and swelling). (C) Initiation of shoot on MS supplemented with BAP. (D) Multi walled carbon nano tubes dispersed in Ultrasonicator. (E) SEM image of FCNT. (F) Graph showing elements of FCNT. (G) Shoot induction on MS supplemented with FCNTs (Functionalised carbon nano tubes). (H) Shoot clusters. (I) Rooted plantlet after 4 months



The present research work studied the effect of BAP, TDZ and NAA in regeneration of successful plant and found that different concentrations and combinations have diverse effects in their growth and development. Several other groups have also used BAP as plant growth regulators for example, Cronauer and Krikorian (1984) carried out micropropagation of banana with the cultivar 'Philippine Lactum' (AAA), 'Grand Naine' (AAA), 'Saba' (ABB), 'Pelipita' (ABB) and found that 5 mg/L BA was the best for shoot multiplication. Similarly Mendes *et al.*, (1999) observed multiplication of shoot tips of cv. 'Naincao' on MS media supplemented with 4.5 mg/L BA. However, Arinaitwe *et al.*, (2000) have also compared various traditional cytokinins (16.8-28.8 μM) and lower concentrations (0.045-9.1 μM) of TDZ for in vitro multiplication of three cultivars ('Kibuzi' (AAA), 'Ndiziwemiti' (ABB) and 'Bwara' (AAA) and found that TDZ as better for multiplication than other cytokinins. Shoot multiplication of 'Ndiziwemiti' progressively increased as the concentration of TDZ increased (9.5 shoots/ explants) whereas the proliferation rate of 'Bwara' and 'Kibuzi' decreased (2-5.4 shoots/ explants) with increasing concentration of TDZ. The use of single plant growth regulator was found to have better response in terms of shoot regeneration than the combinations of growth regulators.

The use of multi walled carbon nanotubes like FCNTs (Functionalised carbon nano tubes) was found to have positive effects in multiplication and successful regeneration. As it was reported in Khodakovskaya *et al.*, (2012) that multi-walled carbon nanotubes enhanced the growth of tobacco cell culture (55–64 % increase over control) in an extensive range of concentrations. In the present study the use of FCNTs in the growth medium was found to be most effective in multiplication and development compared to

all the other treatments evaluated. According to Khodakovskaya *et al.*, (2012), FCNTs (Functionalised carbon nano tubes) improves the root elongation as compared to MS supplemented with growth regulators. Canas *et al.*, (2008) determined the effect of CNTs (carbon nano tubes) on root elongation of six different crop species; tomato, carrot, lettuce, cucumber, onion and cabbage. The improvements in the shoots and roots development may be due to the uptake and accumulation of multi-walled CNTs by roots followed by the movement from roots to leaves (Smirnova *et al.*, 2012). The response showed with the effect of MWCNTs was found to positive response in developing a rapid micropropagation protocol for 'Malbhog' banana.

In conclusion the regeneration of *Musa paradisiaca* 'Malbhog' banana has an increasing demand in North Eastern India, due to its high nutritional value, taste and aroma. Our results show that use of multi walled carbon nano tubes in regeneration is efficient because the MWCNT's used in this study were in range of 12-25 nm (Fig. 1E) which can be inferred that it would be compatible with the general pore size of the plant cell membrane (around 20 μm), and thus be enable them to affect changes by entry into the cells. The results reported here provide novel information on various factors that influence the culture establishment and shoot initiation of malbhog banana. Further future research would be required to study the effects of MWCNT's in shoot initiation at molecular level.

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Abbreviations

BAP: Benzylaminopurine. MWCNT's: Multi walled carbon nano tubes. FCNT's: Functionalized carbon nano tubes. NAA: 1-Napthalenacetic acid. TDZ: Thidiazuron.

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