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RESEARCH ARTICLE

Production of virus-free quality planting material in banana (*Musa spp*.) variety CO 3 (ABB) through meristem culture

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Abstract

The rapid spread of pest and disease infestations significantly affects agricultural productivity and food security. Diseases caused by fungi, bacteria, viruses and other pathogens can severely impact plant health, leading to substantial crop losses. Some crop species, like banana (Musa spp.), transmit viral diseases mostly through vegetative propagules but very rarely through seeds. To address this issue, meristem culture was employed in the present study on the banana variety CO 3 (ABB) to produce virus-free plantlets. Meristem tips, ranging from 20-30 mm in size, were established on full-strength MS medium supplemented with vitamins, ascorbic acid, myo-inositol, 2 mg/L BAP and 0.5 mg/L IAA during the initiation phase. MS medium with 3 mg/L BAP and 0.5 mg/L IAA was optimal for multiple shoot formation, producing an average of 8.5 shoots per explant in 52.5 days. Regarding growth parameters, approximately 3.7 leaves per explant were observed, with an average leaf length of 4.56 cm and an average leaf width of 1.89 cm after the fifth subculture and before the rooting stage. The meristem tips (20-30 mm) of the CO 3 (ABB) variety produced approximately 92.5% virus-free plantlets with a survival rate of 86.5%. The derived plantlets were successfully hardened.

Keywords

banana; CO 3; *in vitro*; micropropagation; meristem; tissue culture

Introduction

Bananas are a rich source of dietary fiber, potassium, vitamin B6, vitamin C and various antioxidants and phytonutrients, all contributing to their numerous health benefits (1). They are commonly grown in tropical and subtropical regions and belong to the *Musa* genus of *Musaceae* family, classified as an evergreen monocot perennial herb (2).

Bananas are the fourth-largest fruit crop globally, following grapes, citrus and apples in production. They are also the most valuable fruit commodity in the world based on export value (3). In 2020, global banana and plantain production reached 119.83 million tons (4). According to FAO (FAOSTAT 2022), banana production increased from approximately 97 million tons in 2008 to nearly 120 million tons in 2020, covering 5.2 million hectares of land (5). India is the top producer of bananas, with a production of 34.53 million metric tons. Within India, Tamil Nadu leads in both area (109.36 million hectares) and production (4,303,918 metric tons), contributing 12% to the nation's overall production (6).

Crop losses from pests have been estimated to range between 20 and 40%, driven by climate change and global trade impacts, which complicate meeting the demands of a growing population (7). The devastating effects of pest infestation are lower fruit quality and yield (8).

The exchange of planting material between countries has facilitated the spread of diseases across regions. To mitigate this risk, it is crucial to implement stringent phytosanitary regulations and index them for viruses before they get into the production chain. These measures ensure the safe exchange of germplasm and enhance effective disease control.

The most economically important viruses affecting bananas are the Banana bunchy top virus (BBTV), several strains of Banana streak Mysore virus (BSMyV), Banana bract mosaic virus (BBrMV) and the recently emerging Cucumber mosaic virus (CMV) (9). In bananas, annual losses due to BBTV amount to approximately $50 million in states like Kerala, consolidated Andhra Pradesh, Maharashtra and Tamil Nadu (10). The BBrMV symptoms include a purplish streak on bracts, pseudostems, midribs, peduncles and occasionally fruits and crop loss due to BBrMV being approximately 30% (11). Consequently, producing virus-free planting material significantly enhances crop health and yield. This study investigates the banana variety CO 3 (ABB), a Karpooravalli x H 201 hybrid, demonstrating higher nematode tolerance than Karpooravalli. The research focuses on using CO 3 banana variety to produce virus-free planting material through meristem culture to obtain a response from the genome ABB.

Materials and Methods

Plant materials

Three-month-old suckers of var. CO 3 (ABB) was collected from the field-grown plants at fruit orchard of Tamil Nadu Agricultural University, Coimbatore and from research farm of ICAR - National Research Centre for Banana, Tiruchirappalli, India. The tissue culture production was conducted at the Tissue Culture Laboratory of the Crop Improvement Division of ICAR-National Research Centre for Banana.

Explant preparation

Outside the laminar hood: The suckers were cut to 10-20 cm in length and rinsed with tap water for 30 minutes before chemical treatment. They were treated with 0.2% cetrimide solution, followed by 0.2% carbendazim and rinsed with Tween-20. Lastly, they were soaked overnight in 0.025% of streptomycin sulfate solution.

Inside the laminar hood: The following day, the suckers were rinsed three times with sterile water before each chemical treatment. They were immersed in 0.1% mercuric chloride for 15 minutes, followed by 4% sodium hypochlorite solution. After 70% ethanol treatment, they were trimmed to 20-30 mm size and initiated in a medium supplemented with growth hormones. The chemical formulation used in the surface sterilization protocol was modified based on previous studies (12) with slight adjustments.

Initiation and multiple shoot induction

The combination of BAP and IAA was previously reported to be effective for shoot multiplication in the meristem culture of bananas (13). In this study, Murashige and Skoog medium (14) with 2 mg/L BAP and 0.5 mg/L IAA was used for initial establishment and 3 mg/L BAP and 0.5 mg/L IAA for shoot multiplication. The cultures were maintained under fluorescent lights with an intensity of 3000 lux at 25°C ± 1°C. Explants were subcultured for 3-4 weeks in fresh medium. The entire tissue culture protocol is depicted in Fig. 1.

Virus indexing

Virus indexing work was carried out at the Molecular Virology laboratory, ICAR-NRCB, Tiruchirappalli, India, to test the samples for the presence of all four major banana viruses (CMV, BBrMV, BBTV and BSMyV). Virus indexing can be conducted at any subculture stage, requiring approximately 150-200 mg of the test sample. In this case, fresh leaf samples were collected from the *in vitro* cultures during the fifth subculture stage before rooting, properly labelled and stored at -20°C until needed for further analysis.

Detection of CMV and BBrMV by TAS- ELISA

The TAS-ELISA test was performed on the meristem plantlets to detect CMV and BBrMV. The polyclonal, monoclonal and goat anti-mouse antibodies were used. Plant samples were ground in liquid nitrogen and mixed with 1 ml of ELISA extraction buffer. The mixture was centrifuged at 8500 rpm for 10 minutes. Two separate 96-well polystyrene ELISA plates (Thermo Scientific, Roskilde, Denmark) were used for CMV and BBrMV, with the layout including two replicates of test samples, healthy controls, positive controls and extraction buffer controls. TAS-ELISA was conducted following the protocol (15) with slight modifications. Each step involving antigen and antibody addition was preceded by washing the plates with 1X PBST wash buffer thrice, each lasting three minutes. The plates were first coated with primary polyclonal (pAb) antibody (IgG) specific to CMV or BBrMV coat protein (CP), diluted 1:500 in coating buffer and incubated for 3 hours at 37°C. Samples were loaded according to layout and incubated overnight at 4°C. The next day, monoclonal antibodies (mAb) for CMV and BBrMV (CP), diluted in conjugate buffer (1:1000), were added and incubated at 37°C for two hours. The secondary polyclonal antibody (GAM-AP), diluted in conjugate buffer (1:2000), was added and incubated for one hour at 37°C. Finally, fresh substrate solution (1 mg of p-nitrophenyl phosphate in 1 ml of substrate buffer) was loaded into plates and incubated at 37°C for 30-60 minutes until a clear reaction was observed. Results were determined by measuring absorbance at 405 nm using a Synergy H1 microplate reader (Biotek, Shoreline, WA, USA). A sample was considered positive if the average optical density (OD) value was at least twice the mean OD of the healthy control.

Detection of BBTV and BSMyV by PCR

DNA extraction from leaf samples: The DNA extraction procedure, which includes key steps *viz.*, precipitation, pelleting and resuspension of DNA, as outlined in a previous study (16), was used to extract DNA from leaf samples of *in vitro* cultures initiated with meristem explants.

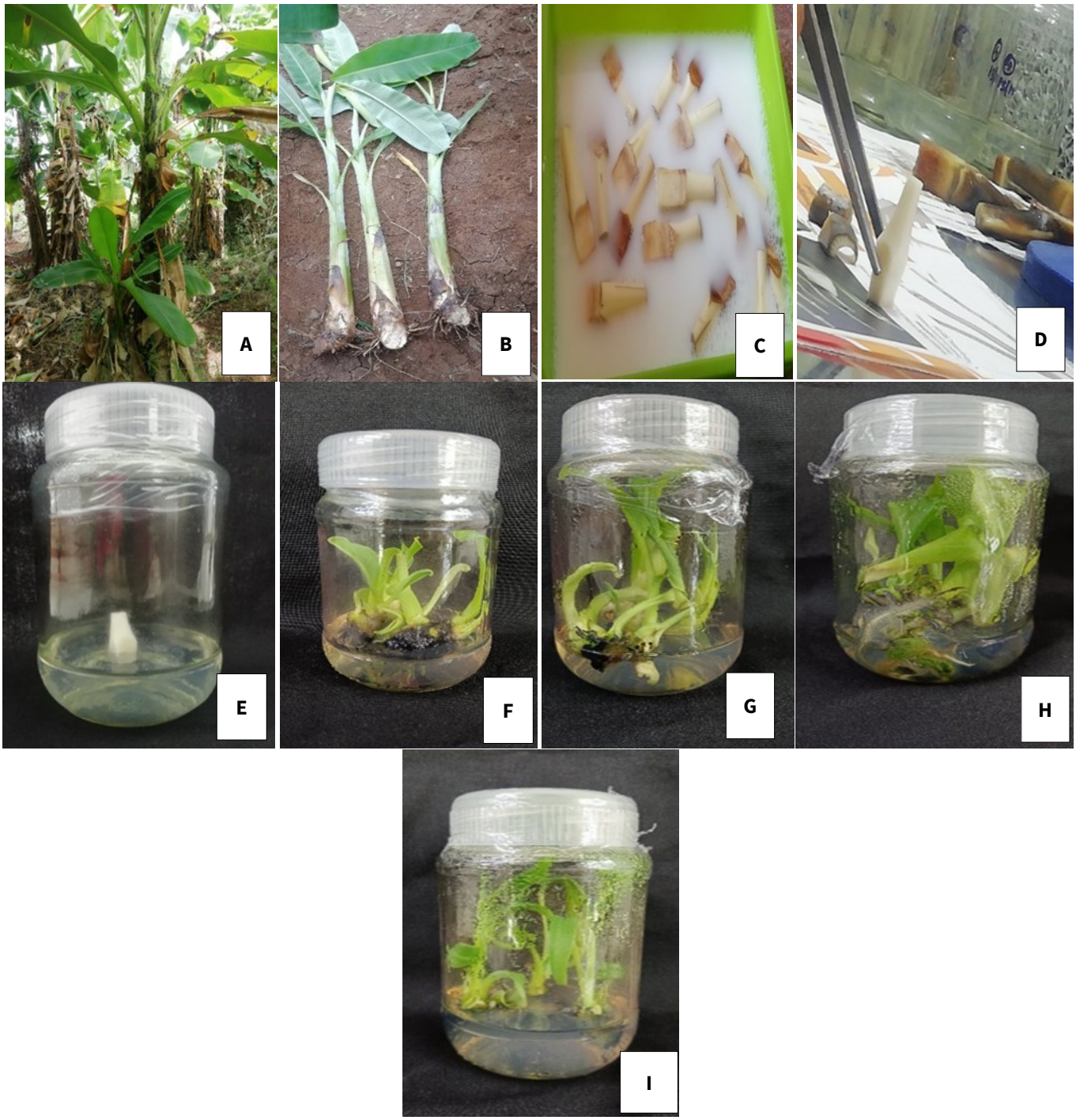

Fig. 1. Stages of banana plant selection and propagation. A) Selection; B) De suckering; C) Sucker treatment; D) Meristem excision; E) Meristem placed in MS medium; F - J) Various subculture stages.

PCR assay: The PCR was carried out using a thermal cycler (Applied Biosystems, USA). The 4 µL of DNA from each sample were pooled before the PCR reaction, with 10 pooled samples used for the PCR process. The entire PCR reaction mixture was prepared in a volume of 20 µL (1 µL of diluted total genomic DNA, 2.0 µL of 10× reaction buffer, 1.5 µL mM dNTPs, 1 µL of respective forward and reverse primer, 0.2 µL Taq DNA polymerase (Takara Bio, New Delhi, India) and volume made up with nuclease-free water. The thermal cycling conditions were as follows: 1) BBTV: 94°C for 4 minutes, followed by 35 cycles of 50 seconds at 94°C, 40 seconds at 53°C, 50 seconds at 72°C and a final extension step 72°C for 10 minutes. Similarly, the cycling condition for 2) BSMyV: 94°C for 3 minutes, followed by 35 cycles of 40 seconds at 94°C, 40 seconds at 52°C, 50 seconds at 72°C and a final extension step 72°C for 10 minutes. The primers used in the study are provided in Table 1. The PCR products were electrophoresed on 1.2% (w/v) agarose gel stained with SYBR green (Thermo Scientific) at 105 V, 400 mA for 33 minutes. The gels were visualized and photographed using gel documentation (GELSTAN Medicare Gel Documentation Systems, India). A 1 kb DNA ladder (Thermo Scientific) was used to determine the sizes of the visible DNA fragments.

Table 1. Details of primers used for the detection of BBTV and BSMyV through PCR assay

Primers used in this study	Sequence of the primers	Size	Reference
BBTV-F	5'ATGGCTAGGTATCCGAAGAAATCC3'	512 bp	(50)
BBTV-R	5'TCAACATGATATGTAATTCTGTTC3'		
BSMysV-F	5'TAAAAGCACAGCTCAGAACAAACC3'	589 bp	(51)
BSMysV-R	5'CTCCGTGATTTCTTCGTGGTC3'		

Table 2. Response of banana var. CO 3 (ABB)

Observations recorded	Response of the var. CO 3 (ABB)
Days taken for greening (average mean)	8.1 d
Days taken for multiple shoot formation (average mean)	52.5 d
Total average number of shoots per explant (after 5 subcultures)	8.5 nos.
Average number of leaves (after 5 subcultures)	3.7 nos.
Average length of leaves (after 5 subcultures)	4.56 cm
Average width of leaves (after 5 subcultures)	1.89 cm
Survival rate	86.5%

Statistical design

The experimental design for the experiment was step-up in a Completely Randomized Design (CRD) with 30 replications (1 sucker per replication). A one-way analysis of variance (ANOVA) was performed. The significance is indicated when means were compared using LSD (Least Significant Difference) and the resulting p-value was less than 0.05. Statistical analysis was performed with the AGRES software.

Results and Discussion

The response of var. CO 3 (ABB) to *in vitro* mass multiplication is summarized in Table 2. Generally, 3 to 4-month-old young suckers were used as they respond better than older ones. The younger explants will exhibit quicker callus differentiation and adventitious shoot formation (12). After establishing meristems under *in vitro* conditions, the milky white meristem changed to greenish color in about 8.1 days. Similar responses were observed in var. Udhayam, where greening occurred within 7 to 10 days (17).

Plant growth regulators are crucial in managing cell division, elongation and shoot proliferation, thereby significantly promoting overall shoot growth (18, 19). The actively dividing meristematic cells respond positively to *in vitro* shoot multiplication due to the stimulatory effect of growth regulators added to the medium (20). The shoot multiplication is significantly influenced by the type and concentration of cytokinins in the medium (21). Among various cytokinins used for shoot proliferation, BAP has consistently shown superior performance across many banana cultivars and varieties (22, 23). Similar results have been obtained in the present study, with 3 mg/L BAP producing a maximum of 8.5 shoots per explant. The enhanced shoot multiplication is due to the uptake and reaction to exogenous cytokinins, besides their endogenous levels (24). The interaction between exogenous cytokinins and endogenous hormones, such as auxins, occurs through a complex signaling network that regulates plant growth and development. When added externally, cytokines influence the cytokinin-to-auxin ratio in the culture medium, promoting shoot initiation and multiplication by activating cytokinin receptors and downstream signaling pathways. Furthermore, the effectiveness of BAP in enhancing shoot proliferation was evident (25).

The adventitious shoot bud formation occurred only after damaging the apical meristems, as reported in previous study (17), which produced more adventitious shoots (26). Multiple shoot formation occurs when the growth medium is supplemented with high cytokinin and low auxin levels (27). Similarly, this study observed multiple shoot bud formation within 52.5 days in a medium containing 3.0 mg/L BAP and 0.5 mg/L of IAA. This might be because of the presence of BAP and IAA in the required ratio (6:1), which would have elevated the endogenous hormonal levels (28), leading to *in vitro* shoot multiplication (29). However, in some recalcitrant varieties like Virupakshi, BAP and IAA, in a higher ratio of 10:1, significantly increased the number of shoots (134.3) per explant over 24 weeks (13). The response to shoot multiplication varied across banana varieties, with AAA-type varieties typically exhibiting better response than ABB types, necessitating the testing of various hormone ratios to identify and standardize the optimal conditions for shoot proliferation for the specific variety. It was observed that rapid shoot proliferation was significantly influenced by the interaction between externally applied auxins and the endogenous auxin levels, demonstrating the importance of balancing external hormone treatments with the endogenous hormonal state (30). This study added IAA at a relatively low concentration of approximately 0.5 mg/L of media. Auxin positively influenced the multiplication ratio, shoot length and diameter at these lower levels. However, when the concentration exceeds optimal levels, it may lead to inhibitory effects, potentially reversing the benefits and hindering plant growth and development (31).

The plant growth regulators are vital for controlling the physiological factors necessary for crop growth and development (32, 33). This study measured various shoot regeneration parameters, such as the number of shoots per explant, shoot length and diameter, leaf count, leaf length and leaf width. The number of shoots produced per explant was 8.5, corroborating the results of a previous study (34), wherein bananas produced 4.8 shoots per explant.

Cytokinin is the primary compound responsible for enhancing *in vitro* regeneration (35). Typically, cytokinins are known to reduce shoot length, but in this case, they increased it (36), registering an average shoot length of 7.1 cm. Higher concentrations of BAP may lead to somaclonal variation, potentially disadvantaging tissue-cultured plants (37). The somaclonal variation refers to genetic changes in plants regenerated from tissue cultures, leading to variations in traits such as size, shape and disease resistance. In banana cultivation, this can result in improved cultivars with better yield, disease resistance, or stress tolerance, but may also introduce undesirable traits.

In the current study, each explant produced an average of 3.7 leaves, measuring approximately 4.56 cm in length and 1.89 cm in width, demonstrating the greater effectiveness of BAP than kinetin in producing better shoot parameters from meristem-derived tissues (38). The present results align with the findings of Cavendish banana studies, where an average of 3 leaves per explant was produced due to a balanced ratio of auxins and cytokinins in the culture medium (39). Similarly, an average leaf count of 5.8 leaves was reported, demonstrating the effectiveness of the growth regulator and coconut water mixture in promoting

leaf development (40). This study successfully determined the optimal composition of the culture medium and cultural conditions to maximize shoot proliferation in the banana variety CO 3 (ABB).

This study used PCR to detect BBTV and BSMyV, whereas TAS-ELISA was for CMV and BBrMV. Nearly 92.5% of the samples were free from all four test viruses (Fig. 2; Table 3). The virus indexing results clearly showed that meristem-derived plants of banana var. CO 3 (ABB) produced plantlets completely free of BBTV, BSMyV and CMV, with only three samples (7.5%) testing positive for BBrMV, producing 92.5% virus-free plants. Many researchers have reported similar results in bananas (13, 34). Meristem cultures are potential explant for producing virus-free planting material in several crops, including figs (41), large cardamom (42), summer squash (43), sweet potato (44), potato (45, 46) and Lilium (47).

For instance, in banana cv. 'Amritsagar,' the elimination percentages for BBTV and BMV-free plants were 57.14% and 64.28%, respectively (48). Similarly, in banana cv. 'Sabri' 57.14% of BBTV-free plantlets were reported (49). With these corroborative findings, meristem culture has been proven to be a highly effective method for producing virus-free plantlets. Bananas were typically propagated through shoot tip culture, which utilizes large-sized explants. The meristematic region, located at the apex and characterized by active cell division, results in higher rates of virus-free plantlets than shoot tip culture. Meristem culture proved to be an efficient method for producing virus-free plantlets. Even in virus-infected plants, combining meristem culture with other *in vitro* virus elimination techniques, such as chemotherapy, thermotherapy, cryotherapy, or electrotherapy, effectively produced virus-free plantlets.

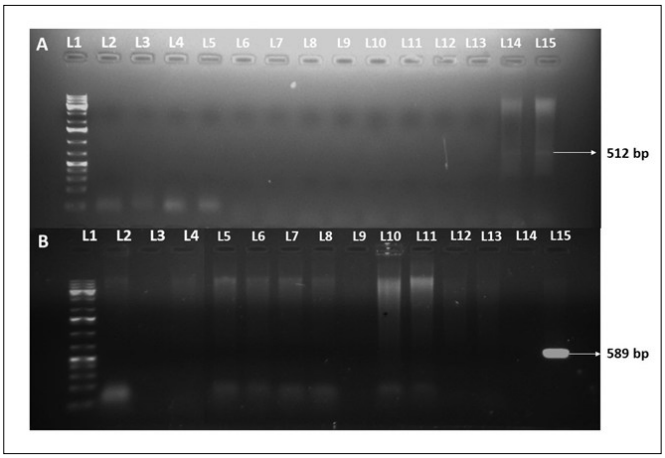

Fig. 2. Agarose gel electrophoresis of PCR product for BBTV and BSMyV in banana variety CO 3 (ABB) (A: BBTV; B: BSMyV) Lane 1: 1 kb DNA ladder, Lane 2 to Lane 11: Test samples of CO 3 (ABB). (A- Lane 12: Negative, Lane 13: Healthy, Lane 14 & 15: Positive). (B - Lane 12 & Lane 13: Negative Control, Lane 14: Healthy, Lane 15: Positive control).

Table 3. Status of virus-free plants in var. CO 3 (ABB)

Total number of plant samples tested	40
Number of positive explants for BBTV (PCR)	0
Number of positive explants for BSMyV (PCR)	0
Number of positive explants for BMV (TAS-ELISA)	0
Number of positive explants for BBrMV (TAS-ELISA)	3
Total number of virus-free explants	37
Percentage of virus-free plants	92.5%

Conclusion

Meristem tip culture has proven effective in producing virus-free planting material in bananas. Culturing meristem tips measuring 20-30 mm in a medium enriched with full-strength MS basal salts, vitamins, ascorbic acid, myo-inositol, 3 mg/L of BAP and 0.5 mg/L of IAA led to their successful multiplication under *in vitro* conditions. The optimized media composition facilitated rapid and efficient shoot multiplication. Using high-quality planting material will enhance banana productivity and yield potential significantly. Meristem culture, as demonstrated in the present study, proved effective in producing healthy planting material, which, in turn, enhanced the productivity of bananas. Meristem culture has been widely applied in other crops as well. Integrating meristem culture and *in vitro* techniques will pave the way for developing advanced methods such as RNA interference (RNAi) and transgenic approaches.

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Authors' Contributions

RS & MSS planned the design of the study. RS, MSS & RA conceptualized and visualized the study. KT conducted the experiment, performed statistical analysis and drafted the manuscript. MSS critically revised and approved final version. VJ & VRB reviewed the manuscript. All the authors approved and read the final manuscript.

Compliance with Ethical Standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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