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Optimized protocol for high-efficiency micropropagation of banana varieties G9 and Malbhog

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ABSTRACT

This research focuses on developing and improving micropropagation techniques for banana shoot tip culture, specifically aimed at enhancing cultivar production. Micropropagation allows for the rapid propagation of plants by cultivating small shoot sections in a controlled laboratory environment. The study investigates various factors, including media compositions, growth regulators, sterilization techniques, and environmental conditions, to optimize the success rate and efficiency of banana shoot tip culture. This work focused on two widely grown banana varieties in Bihar, India—G9 and Malbhog. Researchers adapted the standard Murashige and Skoog basal medium by adding carefully measured concentrations of plant growth regulators. This optimized approach was used to initiate shoot development, promote multiplication, and enhance root formation in the banana plantlets. The protocol also included an effective acclimatization and hardening process using a potting mix of soil. This optimized method resulted in high shoot proliferation rates. The findings contribute to advancing banana cultivar propagation techniques, supporting agricultural efforts to meet the growing demand for bananas, and facilitating the large-scale production of quality banana plants with desirable traits.

1. INTRODUCTION

Bananas (*Musa sp.*) are among the world's most important fruit crops [1]. Originally from tropical regions of South and Southeast Asia, bananas were likely first domesticated in Papua New Guinea. Today, they are cultivated widely across tropical and subtropical regions [2]. Global banana production reaches approximately 40 million metric tons annually. These plants are large perennial herbs, with some species growing as tall as 15 meters. Bananas are monocotyledons and reproduce vegetatively by producing suckers from their underground true stem [3]. This stem supports a false stem made of leaf sheaths, each of which produces a single inflorescence. The female flowers in the inflorescence develop into bananas, either through parthenocarpy or fertilization.

Banana cultivation is widespread across warm regions globally, earning the fruit the nickname "paradise of fruits." India is a major player in banana production, contributing over 26% of the world's supply [4]. In India, bananas are cultivated across diverse climates, from humid tropical and subtropical regions to semi-arid tropical areas. The crop occupies 13% of the country's total agricultural land, accounting for 33% of its overall fruit production. Maharashtra leads in banana production, followed by states such as Karnataka, Gujarat, Bihar, Andhra Pradesh, and Assam [5]. Banana farming plays a crucial role in India's agricultural economy, providing livelihoods to millions of farmers. The fruit is a staple in the Indian diet, consumed fresh or used in various culinary traditions. Bihar is a major banana-producing state in India, with key cultivation areas located in districts such as Vaishali, Samastipur, and Bhagalpur. The state is known for a variety of cultivars, including Dwarf Cavendish, Alpan, Chinia, Chinichampa, Malbhog, Muthia, and Kothia [6].

Among these, Malbhog stands out in the Vaishali district due to its unique and delicious flavor. This cultivar produces large bunches with longer and heavier fruits, making it highly valued in the region. Malbhog bananas, known for their extremely sweet flavor, are often served as a dessert. However, the cultivar faces a severe threat from Panama wilt, caused by *Fusarium oxysporum* which has brought Malbhog to the brink of extinction [7]. Planting Malbhog in new, disease-free areas can help mitigate the impact of Panama wilt, but this is only feasible through micropropagation. This method allows for the large-scale production of high-quality planting material. Moreover, bananas from micropropagated trees tend to have heavier bunches, with more hands and fingers, and a uniform size and shape of the fruit. Another prominent variety is the G9 banana, a *Musa acuminata* cultivar widely grown for its high yield and medium height, making it ideal for commercial farming. G9 is a significant contributor to the Cavendish banana market. It is perfect for commercial agriculture due to its huge fruit yield and typical middle height. Israel introduced this high-yielding cavendish cultivar to India. These are an excellent source of carbohydrates, mostly sugars in ripe banana fruits and starch in unripe ones [8]. The

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farmers in Bihar have embraced advanced cultivation techniques to boost productivity and sustainability, including tissue culture and drip irrigation. The adoption of advanced cultivation methods, such as tissue culture and enhanced irrigation systems, is further increasing production and promoting sustainability in the sector [9].

The production of multiple banana plants is commonly achieved through vegetative propagation and micropropagation techniques [10]. In traditional vegetative propagation, suckers from the parent plant are used, but this method is slow, labor-intensive, and inefficient, yielding only 5–10 suckers per year from a single plant [11]. Additionally, banana production can be significantly impacted by various diseases [12], leading to reduced productivity and lower yields. Micropropagation, on the other hand, involves cultivating banana plants in a controlled laboratory environment using tissue samples. This method enables the rapid production of healthy, disease-free banana plants on a large scale. The key advantage of *in vitro* micropropagation is the generation of genetically uniform plantlets that retain all the desirable traits of the mother plant. Micropropagated plants establish more rapidly, grow at an accelerated rate, have shorter and more uniform production cycles, and produce higher yields compared to traditional methods [13,14]. While micropropagation through shoot tip culture has seen widespread success, variability can still arise due to factors such as genotype, explant type, culture media composition, and phytohormones. These factors can significantly impact the consistency of shoot establishment, and the regeneration of shoots and roots during *in vitro* growth [15].

Despite extensive research on banana micropropagation, the specific requirements of regionally significant cultivars, such as G9 and Malbhog, remain inadequately explored. These cultivars are particularly important for the agricultural economy of Bihar, India. This study aims to systematically optimize hormone concentrations and cultural conditions for these cultivars, addressing a critical gap in existing propagation protocols and providing a practical solution to meet the region's agricultural needs. Consequently, the purpose of this work is to enhance the *in vitro* micropropagation methodology for two types of bananas in a fast and repeatable manner. To do this, the effects of various auxins and cytokinin's at varying concentrations on shoot initiation, multiplication, and elongation as well as *in vitro* shoot rooting are studied. It also investigated how the acclimatization media mix affected the growth of banana plantlets.

2. MATERIALS AND METHODS

2.1. Plant Materials

The study used two widely cultivated *Musa* species in Bihar, G9, and Malbhog, as experimental materials. Healthy sword suckers (weighing around 0.5–1 kg) of these varieties were collected from field-grown plants at the HecureAgro Plants research center in Muzaffarpur, Bihar, which was maintained in disease-free condition. The explants were taken from the lower sections of the pseudostems, containing meristems. The experiment was conducted in the Tissue Culture Laboratory of TPS College, Pataliputra University, Patna.

2.2 Preparation, Sterilization, and Establishment of Shoot Tip Culture

Explants were excised from immature suckers of two banana cultivars. The pseudo stem was cleaned of corm tissue, roots, and leaf sheaths, then thoroughly washed in 0.5% Bavistin solution under running water for 1 hour. The leaf sheaths around the base were removed, leaving only the budding leaves surrounding the protoplast until the shoot tip reached approximately 5 cm. Following a brief rinse in 70% ethanol, the explants were immersed in 2% sodium hypochlorite for 10 minutes, rinsed three times with sterile distilled water, and trimmed to 5 mm in an aseptic environment. The culture medium, a modified Murashige and Skoog (MS) basal medium supplemented with 30 g/l sucrose and solidified with 8 g/l agar, was adjusted to pH 5.7 with NaOH or HCl, then autoclaved at 121°C for 20 minutes. Plant growth regulators, including N6-benzylaminopurine (BAP) at 2, 3, and 4 mg/l and indole-3-acetic acid (IAA) at 0.0, 0.05 and 0.4 mg/l, were added before sterilization. Surface-sterilized explants were placed onto this medium and incubated aseptically for 8 weeks at 25°C ± 2°C under a 15-hour light.

2.3 Shoot Multiplication

Six of the regenerated shoots were aseptically transferred to multiplication media. The same media were sub-cultured six to seven times at intervals to 3 weeks to obtain multiple shoots. The *in vitro*-developed shoot tips were divided into smaller sections, each containing approximately one shoot, for further sub-culturing. The leaves and brown or black basal tissues were removed to expose the meristem, and each section was then transferred to fresh MS media. The number of *in vitro* shoots was increased through multiple sub-culturing steps. Using a sterile scalpel, *in vitro*-proliferated shoots were separated, photoactive shoots were trimmed to a specific size, and each micro shoot was placed on a culture medium supplemented with varying concentrations of BAP and NAA for shoot differentiation.

2.4. *In vitro* Rooting of Shoots

During the initial growth stage, fully grown shoots with enlarged leaves were divided and individually placed in new rooting MS media containing varying amounts of α-naphthalene acetic acid (NAA), IAA, or indole butyric acid. The development of the shoots was evaluated 6 weeks following their transfer to the rooting medium.

2.5. Acclimatization and Hardening of Plantlets

Extended and embedded plantlets (approximately 5 cm in length) were removed from culture vessels, and the agar was gently washed off the roots under running tap water. Plantlets were disinfected with Benomyl (2.0 g/l) for 3 minutes to prevent fungal infections before transplanting. During the acclimatization and hardening phase, plantlets were watered twice a day to maintain consistent soil moisture, ensuring optimal growth conditions. A balanced liquid fertilizer containing nitrogen, phosphorus, and potassium (NPK 20:20:20) was applied at a concentration of 1 g/l every 2 weeks to support root and shoot development. Each plantlet was then individually placed in small polybags filled with a sterile potting mix in a 1:3 (v/v) ratio (sand to soil or press mud). The potting mix was treated with 5% formalin and covered for 3 days, then left to air out for 7 days, with the process completed 10 days before planting. Plantlets were initially placed in a lath house under chambers covered with transparent plastic to maintain high humidity (~85%) for acclimatization and hardening. The humidity was gradually reduced as the plantlets were moved outside the chamber. The survival rate was calculated as the percentage of plantlets that remained viable after 4 weeks of acclimatization. Root development was assessed by measuring the number and length of roots, while shoot height and the number of new leaves were recorded as indicators of growth vigor. Only plantlets meeting these criteria were selected for transfer to larger polybags for further growth. Finally, the plants were transferred to larger polybags containing a mixture of sand, manure, and soil in a ratio of 2:1:1, before being successfully established in the field.

3. RESULTS AND DISCUSSION

3.1. Initiation of Shoots

In the current work, banana shoot tips cultured *in vitro* produced a firm, meristematic ball-like form in initiation media with varying BAP and IAA concentrations. The cultured shoot tip's original creamy white color changed to brown a few days after inoculation. After a period of 4 weeks, the explants' exterior leaf primordia turned green, and a spherical hard coat mass formed, from which adventitious plantlets were produced (Fig. 1a). For each of the two banana varieties, explants cultivated on MS medium supplemented with 2 to 4 mg/l BAP produced the greatest shoot tip initiation response with 100% survival and between 70% and 100% sprout (Table 1). BAP is the cytokinin of choice most frequently utilized in banana tissue culture [16]. A slight variation was observed in the response between the two varieties. For the G9 variety, the highest sprouting percentage (100%) was observed at 3 mg/l BAP with no IAA (3 BAP + 0.0 IAA), which also maintained a 100% survival rate, indicating the suitability of this treatment for optimal shoot proliferation. On the other hand, the Malbhog variety displayed maximum sprouting (100%) with 4 BAP + 0.0 IAA, achieving consistent survival across all treatments except those involving 0.05 mg/l IAA. This indicates that Malbhog responds better to higher BAP concentrations without the inclusion of IAA. Notably, the addition of 0.4 mg/l IAA improved sprouting to 90% in most combinations, suggesting a synergistic effect at this concentration [17]. The results also align with previous reports highlighting the role of BAP in enhancing shoot proliferation in banana tissue culture.

3.2. Multiplication of Shoots

The shoots were moved to the multiplication medium after 8 weeks of culture initiation or once at least one leaf had developed (Fig. 1b). The MS medium supplemented with a mixture of BAP and IAA at doses of 2/0.4, 3/0.05, 4/0.4, and 3/0.05 mg/l yielded the highest shoot multiplication rate (Table 2). The average number of shoots per explant was determined by calculating the multiplication rate for each variety. After five subcultures, the average number of shoots per explant was found to be 265.25 for G9 and 280.20 for Malbhog, respectively. According to reports, the most used PGRs for banana micropropagation are auxins and cytokinin's [18]. In particular, cytokinin's based on adenine is the most widely used cytokinin to influence the rate of shoot multiplication in various species of *Musa* [19]. The G_9 variety proved to be the most fruitful and generated the greatest number of shoots, followed by the Malbhog varieties [20]. The current study used seven cycles of subculturing the shoots to multiply them. During the initial five subcultures, it was observed that the cultures exhibited a higher multiplication rate. Nevertheless, the multiplication rate decreased following the fifth cycle. Subculturing produced several axillary shoots, and 3 weeks following the first subculture, there was a more than three-fold increase in multiplication. After each subculture cycle, another transfer in the same medium increased proliferation by a factor of two to four.

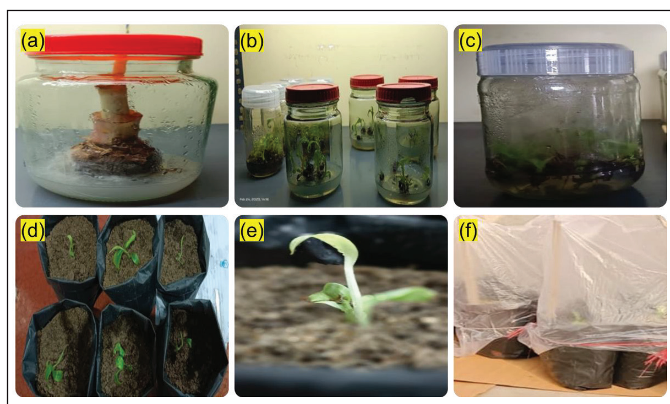

Figure 1. (a) Shoot tip initiation, (b) shoot multiplication, (c) shoot elongation and rooting, (d,e) acclimatization and hardening of plants, and (f) acclimatization inside chamber.

From the table, the shoot multiplication rate for both G9 and Malbhog varieties showed some variability across treatments but remained relatively consistent within the ranges of BAP and IAA concentrations tested. For G9, the multiplication rate varied from 2.33 to 3.75, while for Malbhog, it ranged from 2.69 to 4.20. The mean multiplication rates were 3.19 for G9 and 3.30 for Malbhog, indicating that both varieties responded well to the treatments but with slight differences in optimal hormonal concentrations. Morphological variations, such as thinner shoots or reduced leaf size, might emerge in later subcultures if the hormonal balance is not carefully adjusted [21]. These changes may result from prolonged exposure to high cytokinin levels or an imbalanced auxin-to-cytokinin ratio, affecting plantlet vigor and uniformity. Fine-tuning hormonal concentrations in subsequent

Table 1. Impact of varying concentrations of BAP with IAA, as well as BAP alone, on shoot initiation in two banana varieties.

Treatments (mg/l)	Banana varieties			
	G_9		Malbhog	
	Survival (%)	Sprout (%)	Survival (%)	Sprout (%)
2 BAP + 0.0 IAA	90	80	100	80
2 BAP + 0.05 IAA	100	80	90	70
2 BAP + 0.4 IAA	100	90	100	90
3 BAP + 0.0 IAA	100	100	90	80
3 BAP + 0.05 IAA	100	70	100	90
3 BAP + 0.4 IAA	100	90	90	80
4 BAP + 0.0 IAA	90	80	100	100
4 BAP + 0.05 IAA	90	75	100	80
4 BAP + 0.4 IAA	100	90	90	90

Table 2. *In vitro* shoot proliferation of banana varieties on media with various concentrations of different plant growth regulators.

Treatments (mg/l)	Banana varieties			
	G_9		Malbhog	
	No. of shoots/ explant	Multiplication rate	No. of shoots/ explant	Multiplication rate
2 BAP + 0.0 IAA	2.80	3.75	5.28	3.42
2 BAP + 0.05 IAA	3.28	3.00	3.80	3.33
2 BAP + 0.4 IAA	3.14	3.33	2.28	3.09
3 BAP + 0.0 IAA	3.04	3.09	3.59	4.20
3 BAP + 0.05 IAA	3.60	3.29	3.56	4.12
3 BAP + 0.4 IAA	3.38	3.59	3.87	2.69
4 BAP + 0.0 IAA	3.19	3.19	3.47	2.71
4 BAP + 0.05 IAA	3.90	3.15	2.89	3.14
4 BAP + 0.4 IAA	3.00	2.33	3.23	3.04
Mean	3.25	3.19	3.55	3.30

subcultures is essential to maintain healthy, robust plantlets. For example, at 4 BAP + 0.0 IAA, the multiplication rate for Malbhog declined to 2.71, which may indicate suboptimal conditions affecting shoot quality.

3.3. *In Vitro* Rooting

To promote shoot elongation and basal root formation, strongly developing shoots with enlarged leaves were removed and cultured individually on a fresh rooting medium after every five-cycle subculturing (Fig. 1c). After a few weeks, the basal tufts of roots appeared on all of the transplanted shoots. When individual shoots are placed in a basal medium devoid of PGR, rooting can be encouraged [22]. While basal medium without PGR supported root formation, the addition of NAA provided distinct advantages, including a significant increase in the number and robustness of roots. NAA promotes cell elongation and differentiation, which likely contributed to the thicker, well-branched roots observed in the treated plantlets. On the other hand, auxins cause bananas to initiate new roots [23]. After 6 weeks, growing the shoots on MS medium with 2.45 mg/l NAA for G_9 and 1.60 mg/l NAA for Malbhog types produced the best rooting. In the current study, shoots were transferred to elongation and rooting media in 50 days, at which point they produced roots and were prepared for acclimatization. A similar outcome was reported by Vessey *et al*. [24] stating that root development happened 50 days following shoot transfer.

3.4. Plants Grown *In Vitro* and Field Observed After Acclimatization

Healthy and well-developed *in vitro* grown plantlets, approximately 5 cm in height with three to four leaves, were packed into a small polybag loaded with soil. During the primary hardening process, these polybags were kept in small chambers covered with a plastic for few days (Fig. 1d). After that, plantlets were moved to large polybags that were loaded with soil so they could acclimate (Fig. 1e). The highest vegetative plant development, measured in terms of plant height, pseudo stem girth, and number of photosynthetic leaves per plant, was observed on media containing press mud and sand at a 3:1 ratio (v/v). All of the plants were acclimatized with complete 100% survival. After being successfully established in the field, the hardened plants yielded huge bunches of superior-grade fruits for the medium, comprising combinations of BAP and IAA at 3/0.05 and 4/0.4 mg/l for G9 and Malbhog, can be employed for shoot multiplication. A mortality rate of 12%–18% was observed during the hardening phase. Moreover, when the shoots are cultivated on MS medium with 2.45 mg/l NAA for G_9 and 1.60 mg/l NAA for Malbhog, well-rooted plantlets can be developed. Plantlets that have been toughened and acclimated to potting media containing press mud and sand in a 3:1 (v/v) ratio can achieve the required vegetative growth. Consequently, the current attempt's optimized technique may be applied to the large-scale manufacturing of gigantic banana plantlets (Fig. 1f).

The success of the primary hardening phase can be attributed to the precise control of environmental parameters. High humidity and moderate temperatures minimized water stress, while controlled light levels prevented photodamage and promoted gradual adaptation to external conditions. The gradual reduction of humidity and increase in light intensity during the transition to large polybags helped plantlets acclimate seamlessly, enhancing their overall vigor and field readiness. These findings highlight the importance of tailoring hardening protocols to ensure plantlet survival and growth in subsequent phases. This research opens new avenues for advancing banana micropropagation, with potential applications for other banana cultivars and tropical plants. Future work could explore the genetic stability of propagated plants, ensuring the uniformity and fidelity of desired traits. Additionally, adapting the optimized protocol for a broader range of environmental conditions and refining the acclimatization process could further enhance plant survival rates in diverse growing regions. Finally, integrating molecular tools to monitor plant health and stress response during propagation may provide insights that could improve yield and resilience, supporting the large-scale, sustainable production of bananas and other vital crops.

4. CONCLUSION

This study developed an optimized micropropagation protocol for banana varieties G9 and Malbhog, offering several practical advantages over traditional propagation methods. By fine-tuning the concentrations of plant growth regulators in the modified MS medium for initiation, multiplication, and rooting stages, the protocol achieved high shoot proliferation rates, with an average of 3.25 shoots per explant for G9 and 3.55 for Malbhog, and robust root development. Over five subcultures, the protocol yielded average shoot production rates of 265.25 for G9 and 280.20 for Malbhog per explant, significantly enhancing the efficiency of plantlet production. The acclimatization and hardening process using a sand and soil potting mix ensured high plantlet survival, with a 100% survival rate under field conditions, despite a manageable mortality rate of 12%–18% during the hardening phase. This reliable and scalable method demonstrated greater uniformity in plantlet quality and vigor, reduced variability, and increased the success rate of acclimatization compared to traditional methods. The protocol holds significant potential for supporting large-scale banana cultivation, enhancing crop yields, and contributing to the agricultural economy of the region.

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6. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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