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Standardization for large-scale *in vitro* propagation of banana cv. Robusta

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ABSTRACT

An efficient micropropagation protocol was developed for banana cv. Robusta with shoot tips as explants. The effect of benzyl amino purine (BAP), thidiazuron (TDZ) and indole butyric acid (IBA) on shoot multiplication, elongation and root induction were studied. The highest average number of shoots (8.25 shoots/explant) per explant was obtained in MS medium with TDZ (1.0 mg L^{-1}) and the maximum multiplication rate (9.16 shoots) was obtained after four successive subcultures in MS medium containing BAP (3.0 mg L^{-1}) and TDZ (0.5 mg L^{-1}). GA_3 (0.2 mg L^{-1}) stimulated maximum shoot elongation while IBA (1.0 mg L^{-1}) produced the maximum number of roots. The regenerated plantlets were hardened and successfully established in the field with 90% success rate, showing the same morphological characteristics as the mother plants. The protocol could be used for the commercial production of disease-free Robusta clones by micropropagation.

Key Words - Micropropagation, banana, robusta, phytohormone

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INTRODUCTION

Banana or Musa are one of the most important fruit crops grown worldwide, which are used by more than 400 million people around the world (Pillay & Tripathi, 2007). The new northeastern Koshi region and the ancient Vaishali region of Bihar are the two areas where banana cultivation is most common (Priyanka *et al.,* 2017). These two regions have great climatic conditions for banana cultivation. Robusta (AAA) is one of the most important commercial cultivars of banana grown in Bihar (Kumari & Kumar, 2018). A tasty variety that is utilized for wine and as a vegetable. Due to its impact on the health of people, nutrition is a source for people and hence its importance in trade and commerce, disease free and healthy growth of this fruit is very important. Plant tissue culture is one of the most important means to accomplish this.

Banana *in vitro* propagation is becoming more important globally (Justine *et al.,* 2022). There has been little commercial use of tissue culture technology in this variety, but some simple work has been done. Soil buried sucker as source of explant results in high degree of contamination particularly at the beginning of culture. Therefore, it's crucial to look for alternative tissue sources that could be used as starting materials. It's also advantageous to increase the rate of multiplication by carefully changing some fundamental processes. One of the major problems faced by banana growers is the variability in flowering and productivity. In addition to higher yield potential, tissue grown plants are true to type and have uniform character.

In vitro propagation has several benefits: the high multiplication rate allows for the production of large

numbers of plants in a short period of time, and the uniformity of the plants in terms of their physiology. Moreover, *in vitro* propagation ensures disease-free material throughout the year, irrespective of seasonal variations or environmental conditions (Bhardwaj *et al.,* 2025). Moreover, this method allows the spread of new plant materials to the world quickly, and the exchange and commercialization of new cultivars can be done rapidly around the world. Even though tissue culture plants are commonly undertaken to produce 30% more, it is crucial to critically assess tissue culture plants under field conditions for their genetic consistency. The use of cultured tissue plants in new plantations highlights the need for the establishment of micropropagation protocols for local cultivars cv. Robusta. Due to the lack of cultured tissue plants of this cultivar, it may be replaced by exotic micro propagated cultivars, which could cause its extinction. Thus, it is important to develop a micropropagation protocol for Robusta to conserve this valuable cultivar and to introduce it into new regions.

This biotechnological method, micropropagation continues to be an important tool for the high-quality characteristics of their parent plants. Cloning of several important plant species with high quality of parent plants at large scale are genetically sound and free from pests and diseases. Although it is important, micro propagation research on cv. Robusta has been neglected. Robusta is rare and only one study reported suboptimal multiplication rate. Therefore, an efficient and reliable micropropagation protocol for cv. Robusta is needed to fill this knowledge gap. The present research is designed to meet this need by developing a strong protocol that will allow for the large-scale production of high-quality planting materials and support the cultivation of this valuable variety.

MATERIALS & METHODS

Banana cv. collection of disease-free healthy sword suckers. Robusta was harvested from the farmer's field. The explants were surface sterilized by sequential rinsing with tap water (30 min), 1-2% Teepol detergent solution and 2-3% Savlon solution (5 min) and then rinsed with tap water (5-10 min) in the lab. After that, Explants were pre-treated for 30 minutes in a solution of streptomycin (0.1%), Bavstin (0.1%), ascorbic acid (0.2%) and citric acid (0.4%) in 250 mL of sterile distilled water. Surface sterilizations were done by washing pretreated explants washed twice with sterile distilled water in a laminar flow, then surface-sterilized with 1.0% (w/v) mercuric chloride for 10 minutes. Lastly, they were washed 3-4 times with sterile distilled water under laminar flow. The shoot tip was cut off 2-3 outer layers of the surface-sterilized shoot tip were removed and a 1.0 cm^3 cube containing the apical meristem was excised. The explant was then inoculated onto the selected medium using sterile forceps, under a spirit lamp flame. The culture medium consisted of Murashige and Skoog (MS) basal salts with different concentrations of cytokinins (Table 1) (Figure 1): 6-benzylaminopurine (BAP) and thidiazuron (TDZ). The cultures were grown in a controlled environment chamber at 25 ± 2°C, continuous fluorescent light (2 klx) and relative humidity (RH) 50-80%.

Table 1: Effect of different concentration of BAP and TDZ on *in vitro* culture of banana cv. Robusta, 8 weeks after initial of culture in *in vitro* condition

MS along with plant Growth Regulators (mgL^{-1})		Number of shoots per explant
BAP	TDZ	
1.0	-	2.0
2.0	-	4.25
3.0	-	6.25
4.0	-	5.5
5.0	-	4.25
-	0.5	4.25
-	1.0	8.25
-	1.5	4.25
-	2.0	4.5
-	2.5	2.5
-	3.0	2.33
3.0	0.5	9.16
3.0	1.0	6.43
3.0	1.5	6.43
3.0	2.0	5.61

Figure 1: Explant Initiation

Multiple shoots were differentiated and were subcultured at 6-week intervals to promote further proliferation, multiplication and maintenance of the shoot cultures. Vigorous, differentiated shoots were then cut into individual shoots and placed in a rooting medium. Plantlets were chosen for acclimatization that were healthy. Care was taken to remove banana plantlets from culture bottles, and the medium was carefully washed off. The plantlets were then planted in 1:1 sterilized sand and compost. The plantlets were acclimatized for 30 days in a high humidity acclimatization chamber, followed by a gradual decrease in humidity for primary acclimatization. After primary acclimatization, the plantlets were moved to a greenhouse for secondary acclimatization. Finally, the fully acclimatized banana plantlets were transplanted into the field.

RESULTS & DISCUSSION

Shoot initiation and multiplication effects of BAP and TDZ:

To promote multiple shoots, the explant was planted in MS media containing different levels of BAP and TDZ. Several shoots were produced from the cultured shoot tip of banana (cv. After 6-8 weeks of *in vitro* culture, the growth of Robusta was observed. New multiple shoot buds were seen at the basal margin of the cultured explant, (Figure 2). It was observed that 5-9 shoot per explant was multiplied after 6-8 weeks in multiplication medium using various media and the multiplication efficiency of the shoot was evaluated. The step was repeated three times to test the reliability of the experiment and to get the desired number of shoots. The various concentrations of BAP used for culture initiation and multiplication showed that BAP 3 mg L^{-1} was preferred for higher rate of multiplication (6.25 plantlets/explant; Table. 1). A recent report indicated that the best concentration for shoot regeneration in the banana cultivars Grand Naine and Elakki was the MS medium with a lower concentration (2 mg L^{-1}) of BAP and IAA (1 mg L^{-1}) (Sivakumar & Visalakshi, 2020). The highest multiplication rate (8.25 shoots/explants) was obtained in MS medium supplemented with TDZ 1.0 mg L^{-1} followed by 2.0 mg L^{-1} (4.25 shoots/explants) and 0.5 and 1.5 mg L^{-1} which showed 4.25 shoots/explants, while the lowest number of shoots/explant (2.33 shoots/explant) was observed in MS supplemented with TDZ 3.0 mg L^{-1} (Table 1).

Figure 2: Shoot Induction

The medium containing TDZ was found to have some extent higher shoot multiplication rate than BAP among the two cytokinins tested for multiple shooting. This study revealed that the MS medium supplemented with BAP (3 mg L^{-1}) and TDZ (0.5 mg L^{-1}) was preferred (9.16 shoots/explants) over other concentration combinations used under study for successful multiplication in banana cv. Robusta. Many researchers have reported that BAP was more effective than other cytokinins in promoting shoot initiation and multiplication in different banana cultivars (Arinaitwe *et al.*, 2000; R. M.Z. *et al.,* 2004; Resmi & Nair, 2007; Roels, *et al.* 2005). On the

other hand, it was found that a lower BAP concentration stimulated vigorous growth of the Poovan cultivar (Nair, *et al.* 2018). Although higher BAP concentration had some inhibitory effects on banana shoot growth, discovered by many researchers (Bairu, *et al.,* 2008; Sipen & Davey, 2012; Ferdous, *et al.*, 2015). In banana micro-propagation, use of TDZ at any concentration may result in aberrant and dwarf shoots. This research was later supported in different banana genotypes (Alvard, *et al.*, 1993). The same stunted growth was observed in plants grown in the present study with TDZ medium. As a result, the plants were moved to MS medium with varying GA_3 concentrations (Table- 2).

Table 2- Effect of different concentration of GA3 on *in vitro* culture of banana cv. Robusta

MS along with GA3 (mg L^{-1})	Shoot length (in cm)
0	5.12
0.2	6.90
0.4	6.19
0.6	6.19
0.8	5.59
1.0	5.12

In contrast to our findings, a previous study reported optimal shoot proliferation in banana at a lower TDZ concentration (0.2 mg L^{-1}) in combination with 1.5 mg L^{-1} IAA demonstrated that MS medium augmented with 4 mg L^{-1} BAP and 2 mg L^{-1} IAA optimized multiple shoot production in Grand Naine banana cultivar (Bhaya & Al-Razzaq, 2019; Sivakumar & Visalakshi, 2021; Ahmed & Sharma, 2014). Similarly, Karule *et al.* (2016) reported that MS medium with 10 mg L^{-1} BAP and 1 mg L^{-1} IAA was found to be effective in increasing the number of shoots produced from the shoot tip explants of Virupakshi cultivar.

Banana shoot subculture and shoot elongation

The banana shoot subculture and shoot elongation were performed. After 4-6 weeks of culture, the rhizome buds were subcultured by cutting off the necrotic tissues and then dividing the buds into smaller pieces vertically. Different concentrations of GA_3 were added to MS medium and shoot elongation was encouraged. On MS medium containing 0.4 mg L-1 GA_3, which markedly increased the rate of shoot elongation, optimal shoot elongation was attained (Table- 2). Stunted shoot growth was the outcome of using TDZ as a cytokinin source alone or in conjunction with BAP in this study, suggesting that TDZ may have an inhibitory effect on shoot elongation. Farhani *et al.* (2008) reported that the highest concentration of TDZ was found to be detrimental to shoot formation and promoted abnormal shoot formation, whereas the lowest concentration of TDZ was optimal for shoot induction, in agreement with our results (Htut, 2014).

Effect of rooting media

The rooting started within 15 days after the shoots were transferred to MS basal medium, indicating a relatively rapid onset of rhizogenesis. The mean number of roots per rooted shoot was 5.57 after 4 weeks and the average root length was after 4 weeks. The rhizogenesis and root development were successful with 4.87 cm (Table- 3). IBA was found to be very effective in promoting early rhizogenesis and root development in this investigation, when added to the basal medium, which in turn resulted in a significant improvement in rooting efficiency. The best rooting performance (7.75 roots/plantlet) was obtained with 1.0 mg L^{-1} IBA, which was reflected in the strong root growth that was observed (Figure 3). Likewise, Banerjee & de Langhe, 1985 used IBA to root different cultivars of banana and found it to be an effective rooting hormone for banana tissue culture. But Ahmed & Sharma, (2014), showed that these additives can have a synergistic effect on root induction as they reported that the combination of 200 mg L^{-1} activated charcoal and 1.0 mg L^{-1} IBA resulted in higher rooting efficiency on MS medium. On the other hand, Many Researchers determined that a half strength MS medium with 200 mg L^{-1} activated charcoal and 20 mg L^{-1} adenine sulfate (AS) was best for rooting requirements vary among cultivars, as seen in the rootstocks of Grand Naine and Elakkia (Selvakumar & Parasurama, 2020).

Table 3- Effect of different concentration of IBA on *in vitro* rooting of banana cv. Robusta

Plant Growth Regulators (mgL^{-1}) (IBA)	Number of roots/shoots	Root length (in cm)
0	0	0
0.5	7.45	5.71
1.0	7.75	6.22
1.5	7.24	5.92
2.0	6.63	5.71
2.5	6.83	5.41
3.0	5.20	5.10
Mean	5.57	4.87

Figure 3- *In vitro* Rooting

Figure 4- Hardening and Acclimatization

Hardening and field transfer

After elongation and rooting, the plantlets were carefully removed from the culture vessels and the roots were gently washed with tap water to remove the agar, ready for further transplanting. The survival rate of the regenerated plantlets after field transplantation was high (90%) and the morphological features were similar to those of the mother plants, indicating the effectiveness and genetic stability of the micropropagation protocol. The results of this study showed that the best shoot multiplication rate (9.16 plantlets/explant) was obtained on MS medium with the addition of 3 mg L^{-1} BAP and 0.5 mg L^{-1} TDZ, which indicated that the combination of these plant growth regulators was the most effective for the maximum shoot proliferation. The 0.4 mg L^{-1} GA_3 and 1 mg L^{-1} IBA in MS medium were found to be optimum for shoot elongation and root proliferation, respectively. On the other hand, higher TDZ concentrations in MS medium increased shoot multiplication, while at the same time reducing shoot elongation, suggesting a trade-off between these two parameters. Interestingly, the individual application of TDZ or BAP did not produce better results in terms of plantlet production in Robusta, while the synergistic effect of higher BAP concentrations with lower TDZ concentrations was more effective in improving plantlet regeneration.

CONCLUSION

Optimization of plant growth regulators (PGRs) showed that 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} TDZ was the best combination for shoot proliferation in banana cv. Robusta. On the other hand, in vitro culture development for shoot proliferation decreased with concentrations of IBA > 0.5 mg L^{-1} and BAP > 3.0 mg L^{-1} in MS. In addition, the supplementation of 0.2 mg L^{-1} GA_3 to MS medium resulted in maximum shoot elongation, while 1.0 mg L^{-1} IBA medium gave the highest number of roots and thus it could be used as a viable protocol for commercial-scale plantlet regeneration of banana cv. Robusta.

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