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In vitro Plant Regeneration of Banana cv. Bhimkol (*Musa balbisiana*, BB) through Shoot Tip Culture

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Bhimkol (*Musa balbisiana,* BB) is a wild diploid seeded cultivar, belonging to the family Musaceae. It is tolerance to pest and diseases, since it has high demand for commercialization for its high nutritive values. Therefore, the present study was undertaken to standardize the protocol for *in vitro* plant regeneration *via* shoot tips for further breeding programmes (*in vitro* mutagenesis). The efforts were made to improve the shoot proliferation efficacy through MS medium enhanced with different combinations of cytokinins (BAP and TDZ) and auxins (NAA) to achieve higher number of multiple shoots with shorter period. Among the different combinations of BAP, TDZ and NAA, T_5 (MS + TDZ 0.1 mgL^{-1} + NAA 0.2 mgL^{-1}) was recorded early response (6.60 days), percentage of explant response to initiation (93.33%), days taken for multiple shoot induction (46.40 days) and the highest

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number of shoots per explant (8.93). The days taken for rooting, number of roots per shoot and highest root length was noted in the treatment half strength MS + 1.0 mgL^{-1} IBA + 1.0 g L^{-1} activated charcoal.

Keywords: Musa balbisiana; in vitro regeneration; shoot tip; Bhimkol; multiple shoots.

1. INTRODUCTION

Bananas and plantains are vital foods throughout the tropical and subtropical regions of the world. The *Musa acuminata* (AA) and *Musa balbisiana* (BB) are two species from which edible bananas are derived [1]. Banana production is hampered by many diseases, pests and traditional propagation approaches. India is the largest producer of bananas with production of 31.5 million tonnes from an area of 0.878 million hectares with an average productivity of 35.8 tonnes ha^{-1} [2].

Bhimkol is an important backyard crop distributed mostly in Assam and to some extent to neighbouring states like West Bengal, Arunachal Pradesh, Nagaland, Meghalaya, *etc.* It has been exploited as a highly nutritive source of energy, vitamins and amino acids. Bhimkol fruits are consumed as a dietary supplement by the people of Assam and parts of North-east India, because fruits are rich in carbohydrates, vitamins and proteins. Fresh ripe fruit pulp has antiperoxidative and antioxidant characteristics that can help to prevent oxidative stress-related illness [3]. It is regarded as an essential baby weaning food due to its nutritious sweet pulp. The various portions of the plant are used as food, in religious rites and also as medication to treat diseases such as jaundice and dysentery. Therefore, this plant has farfetched commercial value in this area [4]. Bhimkol banana has many advantages but not gain more popularity due to long crop duration (740 days) and presence of more number of seeds (100-150) which are compactly arranged in the fruit and it is the most undesirable character not only from the consumption point of view but seeds of Bhimkol hinders during processing for product development (Northeast news, 30th April, 2014). To induct the desirable traits like seedlessness, dwarfness and early flowering, in this cultivar is very important for commercialization of this crop. It needs standardized *in vitro* protocol for further breeding studies such as *in vitro* mutagenesis in different explants.

The corms and small sword suckers were used as traditional propagation method [5,6]. However, the conventional propagation plant material is not ideal propagule, since they transmit weevils, fungal pathogens, nematodes and viruses [6,7]. Hence, shoot tip culture has become increasingly popular in several countries since 1985 (Israel, the Canary Islands, Taiwan and South Africa) as a substitute to conventional planting material [8].

Micropropagation technique offers high rate of multiplication, genetically uniform, pest and disease-free planting materials, year around availability of plant material, short harvest interval compare to conventional planting material and faster growth in the early growing stages [9,10,6]. In banana micropropagation different explants sources and approaches are used by several authors [11-17]. *In vitro* multiple shoot formation is regulated by cytokinins and the optimal dosage depends on genotype [18,19]. Plant growth hormones are vital medium components in tissue culture for defining plant cell development. 6-benzylaminopurine (BAP) and kinetin are well documented cytokinins used in banana tissue culture to reduce apical dominance and encourage both axillary and adventitious bud formation from meristematic tissues [11].

Apart from the influence of genotypes, shoot proliferation rate and elongation are affected by cytokinin types and their concentration. Adenine-based cytokinins are used in several *Musa* spp. for *in-vitro* multiplication of shoots. 6-benzylaminopurine (BAP) is the most frequently preferred cytokinin [5,9]. The concentration of exogenous cytokinin appears to be the main factor affecting multiplication. However, the use of diphenyl urea derivatives (thidiazuron) in *Musa* shoot-tip culture is very occasional. The cultivars responded significantly better in their shoot proliferation responses to TDZ than BAP and that TDZ was more economical than adenine-based cytokinins.

However, there are no reports on Bhimkol commercial scale micropropagation, which commonly found in North-East regions of India. Only few reports are available on micropropagation of *M. balbisiana* (BB). Therefore, the attempts were undertaken in this study to mass multiply the banana cv. Bhimkol.

In the present study, the potential of rapid clonal propagation of *Musa balbisiana* (BB) cv. Bhimkol was studied. The impact of BAP and TDZ combinations on shoot proliferation and influence of MS medium, charcoal, IBA and NAA on rooting were also investigated.

2. MATERIALS AND METHODS

2.1 Explant Source

Musa balbisiana (BB) cv. Bhimkol plants were grown at College Orchard, Horticultural College and Research Institute, TNAU, Coimbatore, India. The two to three months old healthy and vigorously grown sword suckers were used as explant and the explants were collected from well-maintained mother block for the current study.

2.2 Preparation of Explants

The suckers were properly cleaned in running tap water and then washed in soap water solution (Tween-20) for 30 minutes, to remove the adhering of soil particles. The outer leaf sheaths, leaf bases, roots and rhizome tissues were trimmed using stainless steel knife, until the dimension of the shoot was 4 to 6 cm. The shoots were immersed in 1% Carbendazim (Fungicide) solution for 60 minutes and were then washed in running tap water. Again, trimmed the shoots and were later immersed in Carbendazim 0.5% and Streptomycin 0.05% for 2 hours to avoid the surface fungal and bacterial contaminants. Then the explants were rinsed well with distilled water. Afterwards explants were trimmed and kept in solution containing cetrimide (0.5%) for 30 minutes and rinse with distilled water. The washed explants were kept in solution containing ascorbic acid (100 mgL^{-1}) and citric acid (150 mgL^{-1}) for 30 minutes and rinse with double distilled water.

2.3 Surface Sterilization and Inoculation of Explants

Explants (pale white tissues) were taken to the laminar air flow hood and disinfect with ethanol for 30 seconds, afterwards rinsed 3-4 times with sterile distilled water. Again, the explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$, SRL, Pvt. Ltd., India) for 10 minutes then finally washed with sterile distilled water for 4-5 times. Then the explants were transferred to the sterilized glass petri plates (150 mm × 15 mm, Borosil®, India) and the shoot tips (1 cm) were inoculated on 15 ml of MS liquid medium held in autoclaved test tubes (50 mm × 25 mm Borosil®, India).

2.4 Nutrient Media

The multiplication medium contains MS basal salts [20], adenine sulphate 75.0 mgL^{-1} (Hi-media, India), ascorbic acid 100.0 mgL^{-1} (Hi-media, India) sucrose 30.0 gL^{-1} (Hi-media, India), clarigel 2.5 gL^{-1} (Hi-media, India), BAP - 3.0, 5.0 and 7.0 mgL^{-1} (Hi-media, India), TDZ - 0.1, 0.2 and 0.3 mgL^{-1} (Sigma-Aldrich, USA) and with 0.2 mgL^{-1} NAA (Hi-media, India). The medium of the pH was adjusted to 5.8 with 0.1 N sodium hydroxide (NaOH) or hydrochloric acid (HCl) prior to the addition of the gelling agent and autoclaving. The medium was autoclaved for 20 minutes at 121ºC at 15 psi. All growth regulators were filter sterilised and added after the media had been sterilised.

2.5 Culture Conditions

The aseptic cultures were incubated at 27±2 ^{0}C under 16/8 h (light/dark) photoperiod for further observations. Cultures were observed daily for the first 15 days and then at weekly intervals to record contamination (if any).

2.6 Rooting and Acclimatization of *in vitro* Regenerated Plantlets

For *in vitro* rooting, the regenerated shoots were cultured in ½ Ms medium added with 0.5 mgL^{-1} NAA and 1 gL^{-1} activated charcoal were used. The well *in vitro* regenerated shoots with healthy roots were transferred to the cocopeat medium in pro-trays as primary hardening and the plants were kept in 50% shade net house for 30 days. After those plants were transferred to the poly bags contains potting mixture (1:1:1, soil:sand:FYM) as secondary hardening and the plants were kept in 50% shade net house.

2.7 Observations and Data Analysis

The days taken for response (greening), percentage of explant response to initiation of shoot tips were recorded at initial phase. The days taken for multiple shoot induction, total number of shoots, shoot length and number of leaves were recorded during third sub culture of shoot multiplication stage. The experiment was laid in a completely randomized design (CRD),

with three replications of 5 explants per treatment. The data were subjected to ANOVA as suggested by Panse and Sukhatme [21]. The data were analysed using statistical package SPSS (Statistical Package for Social Studies) statistics version 22.0. Mean values were separated by Duncan's multiple range test (DMRT) at 5% probability level.

3. RESULTS

3.1 Initial Culture Establishment

Shoot tip is used as an explant for the current study and the cultures were initially established in the MS liquid medium containing BAP (2.0 mgL^{-1}) and NAA (0.2 mgL^{-1}). The explants free from contaminants resulting in the establishment of aseptic cultures and the explant responds within one week, the colour changes from creamy white to greenish colour (greening of shoot tip) indicating successful establishment of the culture. These cultures were used for the *in vitro* shoot proliferation and medium is supplemented with various levels of BAP, TDZ and NAA.

The effect of different combinations of BAP and TDZ on shoot proliferation with NAA were investigated. The treatment showed significant results in early response (greening) which was observed within a one week in T_5 (6.60 days) followed by T_4 (7.80 days) and T_6 (7.93 days). The delayed greening was observed in T_1 MS basal medium (11.07 days). The percentage of explant response to initiation was recorded highest in T_5 (93.33 %) followed by T_4 (90.67 %) and lowest percentage observed in T_1 (66.67 %) (Table 1).

3.2 Combinations of Growth Hormones on Shoot Proliferation

The days taken for multiple shoot initiation was documented at the end of the third sub culture (Table 1). The MS medium enhanced with TDZ 0.1 mgL^{-1} + NAA 0.2 mgL^{-1} showed earlier multiple shoot induction (46.40 days) followed by BAP 7.0 mgL^{-1} + NAA 0.2 mgL^{-1} (46.87 days) which both were on par and the maximum days taken for multiple shoot induction in T_1 (62.87 days). The highest number of shoots per explant were produced in T_5 (8.93) followed by T_4 (7.07) and the least number shoots per explant were observed in control (2.60) (Table 2).

The highest shoot length was observed in BAP 7.0 mgL^{-1} + NAA 0.2 mgL^{-1} (5.60 cm) followed by T_2 and T_3 (5.27 and 5.26 cm respectively) and the lowest shoot length was observed in T_7 (4.13 cm). The number of leaves were recorded at the end of third sub culture (Table 2). The maximum number of leaves were produced in T_3 (4.27) followed by T_2, T_5, T_4 and T_6 (4.0, 4.00, 3.93 and 3.87) these treatments are on par. Lesser number of leaves were produced in control (3.0).

Table 1. Influence of growth regulators on days taken for greening and days taken for multiple shoot induction in banana cv. Bhimkol

Treatment		Days taken for greening	% of explant response to initiation	Days taken for shoot induction
T_1	MS basal medium (Control)	11.07^{a*}	66.67^{e}	62.87^{a}
T_2	MS + BAP 3.0 mgL^{-1} + NAA 0.2 mgL^{-1}	8.80^{b}	72.00^{d}	56.47^{b}
T_3	MS + BAP 5.0 mgL^{-1} + NAA 0.2 mgL^{-1}	8.07^{c}	86.67^{b}	51.53^{c}
T_4	MS + BAP 7.0 mgL^{-1} NAA 0.2 mgL^{-1}	7.80^{c}	90.67^{a}	46.87^{d}
T_5	MS + TDZ 0.1 mgL^{-1} + NAA 0.2 mgL^{-1}	6.60^{d}	93.33^{a}	46.40^{d}
T_6	MS + TDZ 0.2 mgL^{-1} + NAA 0.2 mgL^{-1}	7.93^{c}	84.00^{b}	48.20^{d}
T_7	MS + TDZ 0.3 mgL^{-1} + NAA 0.2 mgL^{-1}	$8.27b^{c}$	78.67^{c}	48.13^{d}
	SEm±	0.19	1.21	0.71
	CD = 0.05	0.58	3.66	2.17
	CV (%)	3.97	2.56	2.40

**Mean of four replications. Values followed by a common letter are not significantly different at the 5% - level by DMRT*

Table 2. Influence of growth regulators on number of multiple shoots/explants, shoot length (cm) and number of leaves in banana cv. Bhimkol

Treatment		No. of multiple shoots	Shoot length (cm)	No. of leaves
T_1	MS basal medium (Control)	2.60[e*]	4.62[b]	3.00[c]
T_2	MS + BAP 3.0 mgL^{-1} + NAA 0.2 mgL^{-1}	4.13[d]	5.27[a]	4.00[ab]
T_3	MS + BAP 5.0 mgL^{-1} + NAA 0.2 mgL^{-1}	5.53[c]	5.59[a]	4.27[a]
T_4	MS + BAP 7.0 mgL^{-1} NAA 0.2 mgL^{-1}	7.07[b]	5.60[a]	3.93[ab]
T_5	MS + TDZ 0.1 mgL^{-1} + NAA 0.2 mgL^{-1}	8.93[a]	4.31[bc]	4.00[ab]
T_6	MS + TDZ 0.2 mgL^{-1} + NAA 0.2 mgL^{-1}	5.00[cd]	4.23[c]	3.87[ab]
T_7	MS + TDZ 0.3 mgL^{-1} + NAA 0.2 mgL^{-1}	4.20[d]	4.13[c]	3.47[c]
	SEm±	0.29	0.12	0.22
	CD = 0.05	0.89	0.36	0.66
	CV (%)	9.46	4.30	10.04

**Mean of four replications. Values followed by a common letter are not significantly different at the 5% - level by DMRT*

Table 3. Effect rooting h media on root parameters of banana cv. Bhimkol

Treatments		No. of days taken for rooting	No. of roots	Root length (cm)
RM_1	½ MS (Control)	14.80[a*]	3.20[e]	4.30[e]
RM_2	½ MS + 0.5 mg/L NAA + 1g/L AC	13.05[b]	4.25[d]	5.14[d]
RM_3	½ MS + 1.0 mg/L NAA + 1g/L AC	13.10[b]	4.75[c]	6.21[b]
RM_4	½ MS + 0.5 mg/L IBA + 1g/L AC	12.60[b]	5.35[b]	5.76[c]
RM_5	½ MS + 1.0 mg/L IBA + 1g/L AC	10.35[c]	6.50[a]	10.34[a]
	SEm±	0.20	0.12	0.12
	CD = 0.05	0.61	0.38	0.38
	CV (%)	3.14	5.18	3.93

**Mean of four replications. Values followed by a common letter are not significantly different at the 5% - level by DMRT*

Fig. 1. Steps involved in shoot tip culture of banana cv. Bhimkol

A. Sword suckers for preparation of explants
B. Trimming of explants for soap water treatment
C. Explants immersed in fungicide (Bavistin 1%) for 1 hour
D. Trimmed and explants immersed in streptomycin (0.05%) for 2 hours
E. Explants dipped in cetrimide solution (0.05%) for 30 minutes
F. Trimmed and ready for surface sterilization ($HgCl_2$ 0.1% for 10 minutes)
G. Initiation of explant on MS medium with 5 mg/L BAP
H. Response of explant after 7 days of initiation

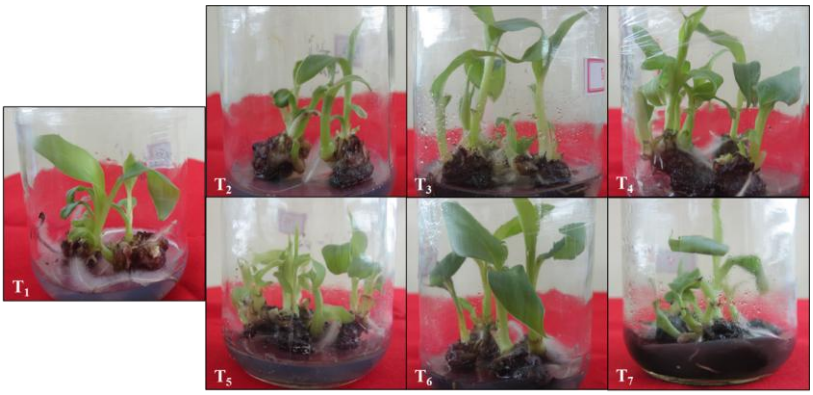

Fig. 2. Effect of cytokinins on shoot multiplication in banana cv. Bhimkol

T_1 : MS basal medium (Control)
T_2 : MS + BAP 3.0 mgL^{-1} + NAA 0.2 mgL^{-1}
T_3 : MS + BAP 5.0 mgL^{-1} + NAA 0.2 mgL^{-1}
T_4 : MS + BAP 7.0 mgL^{-1} NAA 0.2 mgL^{-1}
T_5 : MS + TDZ 0.1 mgL^{-1} + NAA 0.2 mgL^{-1}
T_6 : MS + TDZ 0.2 mgL^{-1} + NAA 0.2 mgL^{-1}
T_7 : MS + TDZ 0.3 mgL^{-1} + NAA 0.2 mgL^{-1}

Fig. 3. Effect of NAA and IBA on rooting of banana cv. Bhimkol

RM_1 : ½ MS (Control)
RM_2 : ½ MS + 0.5 mg/L NAA + 1g/L AC
RM_3 : ½ MS + 1.0 mg/L NAA + 1g/L AC
RM_4 : ½ MS + 0.5 mg/L IBA + 1g/L AC
RM_5 : ½ MS + 1.0 mg/L IBA + 1g/L AC

3.3 Rooting and Acclimatization of *in vitro* Shoots

Half strength MS medium fortified with 1.0 mgL^{-1} IBA produced earlier roots (10.35 days), higher number of roots (6.50) and highest root length (10.34 cm) followed by 0.5 mgL^{-1} IBA. The delayed rooting (14.80 days), least number of roots (3.20) and lowest root length (4.30) was observed in control (Table 3). The well-developed rooted shoots were planted in pro-trays containing sterilized cocopeat. After 30 days, the primary hardened plantlets were planted in polythene bags containing sand, soil and FYM (1:2:1) and maintained in 50% shade net house.

4. DISCUSSION

Banana is threatened by several devastating diseases and pests transmitted by planting material and in the present circumstances Fusarium wilt is the most important problem in banana cultivation across the world. Cultivated bananas are diploid, triploid and some are tetraploids with genome *Musa acuminata* (AA) and *Musa balbisiana* (BB) [22]. All genomic groups do not respond equally for plant tissue culture. The cultivars specifically those with genome *balbisiana* are shows poor response [23].

Cytokinins are the most significant growth hormones in the plant tissue culture and are frequently used in several *Musa* spp. for *in vitro* proliferation. Growth and multiplication rate is enormously dependent on function of cytokinin concentrations [24]. 6-benzyl aminopurine (BAP) is the ideal cytokinins used in a banana micropropagation whereas, TDZ is used in rare cases [14,25,26]. Therefore, the present investigation was examined to standardize the

protocol for micropropagation in banana cv. Bhimkol with various concentrations of cytokinins (BAP and TDZ).

Thidiazuron (TDZ) is a synthetic diphenyl urea derivative of cytokinin group and superior to the BAP [27] in overcoming apical dominance and stimulates the lateral bud break and multiple shoot induction. In the present study, cytokinins *viz.,* BAP and TDZ along with NAA were tested at different levels for shoot proliferation. As previous research, the synergistic impact of cytokinins and auxins may have stimulated the shoot bud production in recalcitrant bananas [24,17,28]. This may be due to suppression of an apical dominance, which might have led to improved axillary bud formation and restricted growth of explants as opined by Huetteman and Preece [29]. The ability of TDZ might be associated to the increase in biosynthesis of endogenous adenine based cytokinins and its least susceptible to the degrading enzymes present in the plant system [29,30]. The rate of multiple shoots in banana is function of cytokinin in the culture media [31]. Further, TDZ has a higher *in vitro* efficiency because it is resistant to degradation by all cytokinin oxidase enzymes [32].

The number of shoots gradually decreases with an increase in TDZ concentration and also it retards the shoot elongation [33,34,35]. The highest shoot length was observed in medium fortified with BAP 5.0 mgL^{-1} in banana and the same findings were reported by Meitei et al. [36] Rahaman et al. [37]. The present study revealed that, due to the compaction of the shoots in medium supplemented with TDZ regenerated plantlets had produced lesser number of leaves. Similar findings were reported by Rabbani et al. [38] and Rahaman et al. [37] in banana (*Musa* spp.).

For sustained growth and development, shoots regenerated from *in vitro* cultures have appropriate root numbers and root length prior to transfer to the *in vivo* environmental conditions. Most commonly used rooting hormones *viz.,* IBA, IAA and NAA to induce rooting in *in vitro* regenerated shoots of banana [39,40,41]. The half strength MS medium supplemented with different levels auxins like NAA and IBA were tested for root initiation and root growth. There was a corresponding increase in the number of roots and root length, as the increase in the concentrations of IBA and NAA [42].

When the IBA levels was higher over 1.5 mg^{L-1}, there was an inhibitory impact. This is consistent with the results of Lohidas and Sujin [43], who demonstrated that IBA has an inhibitory impact at doses over 2 mgL^{-1}. The 0.1% activated charcoal was added in the rooting medium and it promotes the rooting in banana reported earlier [44,45]. This might be because it results in the permanent adsorption of inhibitory substances and a reduction in the build-up of noxious metabolites such as phenolics, thus encouraging plant growth in addition to rooting [46].

5. CONCLUSION

Based on the current investigation resulting in the development of an effective *in vitro* regeneration of banana cv. Bhimkol (*Musa balbisiana*, BB), allowed to production of true to type planting material. These findings suggested that the methodology is very beneficial for farming community for large scale production of planting materials of Bhimkol cultivar *via in vitro* regeneration.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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