

Original Research Article

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High *in vitro* Shoot Multiplication for Efficient Micropropagation of Banana Cv. Robusta (AAA)

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ABSTRACT

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The cultivar Robusta forms the mainstay of commercial banana cultivation in India. An efficient micropropagation system with high multiplication rate will boost banana cultivation and assist banana based industries in the country. The shoot apices of banana cv. Robusta were cultured on two basal media MS and B5 supplemented with different concentrations and combinations of plant growth regulators (PGR). The tissue culture responses were observed for shoot multiplication and effect of subculture on rate of shoot multiplication till 8th subculture. The maximum frequency of multiple shoot formation (97.95%) and number of differentiated shoots per culture (27.25) was achieved on medium M11 (MS+1.14 μ M IAA+19.97 μ M BAP). The medium was further used for the multiplication of shoot buds upto eight subculture cycles. The mean number of differentiated shoots per culture was maintained upto 5th subculture cycle thereafter declined in 6th (-16.67%) to 8th (- 40.35%) subcultures. The *in vitro* developed shoots were rooted on medium M17 (MS + 4.92 μ M IBA). The well rooted plantlets were acclimatized to field condition.

Introduction

Robusta (AAA) is one of the most important commercial cultivar of banana grown in Asia-Pacific. It is a clone of the dominant Dwarf Cavendish cultivar and covers majority of the area under cultivation of banana in India because of many superior features such as pleasantly flavoured, attractive colour, pulpy fruits, exportable large bunches and robust pseudostem which can withstand strong winds (Ghosh *et al.*, 2009). It is popular with other names as Poya, Valery or Tall Mons Mari in

other countries (Simmonds, 1982). Robusta is the main cultivar grown in Koshi region of Bihar.

Cultivar Robusta is conventionally propagated through suckers with low multiplication rate. The propagules carry pest and pathogen and the resultant plants require longer period for flowering and fruiting. Micropropagation helps in overcoming the problems of conventional propagation and allows regeneration of large population of disease free quality plants in a short period of time for

planting in new areas. Further, micropropagated plants are healthier, early maturing and bear longer bunch with larger fruits in higher numbers.

There is lack of a proficient micropropagation system utilizing shoot apices in the cv. Robusta. There are few reports with low multiplication rates (Vani and Reddy, 1999; Senthilkumar and Ramsundar, 2009; Choudhary *et al.*, 2014). Thus, the objective of the work is to develop an efficient dexterous micropropagation system for cv. Robusta to enhance production of quality propagules with less time and effort.

Materials and Methods

Collection of disease free healthy sword suckers of banana cv. Robusta was done from the experimental field of the Department of Horticulture, Dr. Rajendra Prasad Central Agricultural University, Pusa, Bihar, India. The collected explants were then prepared and pretreated as suggested by Kumari and Kumar (2016). The prepared explants were brought to laminar air flow after washing with distilled water. A solution of 0.2% HgCl₂ was applied to the explants for their surface sterilization. The traces of HgCl₂ solution was removed by three consecutive washing of the explants with sterile distilled water.

The prepared explants were further trimmed to a size of 0.5 cm³ cube containing apical meristem and inoculated individually onto the selected medium, which consisted of MS and B5 basal media supplemented with different concentrations and combinations of plant growth regulators (Auxins- 2,4-D, IAA and IBA; and Cytokinins- BAP, KIN and TDZ) (Table 1) (Figure 1A). The cultures were incubated in culture room at an ambient temperature of 25 ± 2°C, with continuous fluorescent light of about 2 kilo lux intensity and relative humidity (RH) 50 to 80 %.

The differentiated multiple shoots were routinely subcultured after 6th week for further proliferation, multiplication and maintenance. They were divided into groups of 3-5 shoots and inoculated into individual culture bottles. After sufficient propagule multiplication, preferably in sixth subculture, the proliferated healthier differentiated shoots were further divided into single shoots and inoculated into rooting medium for development of roots to acquire a complete plantlet. The well developed tissue cultured plantlets having healthy shoot and roots were selected for acclimatization. The banana plantlets were removed cautiously from culture bottles. The adhered medium from the roots of the plantlets were gently removed by washing with a soft brush and transferred to a mixture of sterilized sand and compost (1:1) individually in plastic pots. The plantlets were initially kept under high humid conditions in an acclimatization chamber for primary acclimatization in progressive way for 30 days. The primary acclimatized plantlets of banana were then transferred to green house for secondary acclimatization. Acclimatized banana plantlets were subsequently transferred to field.

The *in vitro* responses were observed and recorded at progressive stages. The experiments were set up in a completely randomized design (CRD) with a minimum of 30 cultures per treatment. All the data were analyzed in CRD by executing one factor analysis of variance (ANOVA) using OP Stat. The means were compared using Duncan's multiple range test (Duncan, 1955) to find the difference at 5% (P<0.05) level. The results were expressed as mean ± SE of four replications.

Results and Discussion

The multiple shoot differentiation from the cultured shoot apices of banana cv. Robusta

was observed after twenty to thirty days of culture. New multiple shoot buds were observed proliferating from the basal margin of the cultured explant (Figure 1B). The extent of shoot multiplication was evaluated on the basis of the frequency and the number of differentiated shoots per culture. The frequency of multiple shoot formation ranged from 26.43 to 97.95% and the number of differentiated shoots per culture from 7.25 to 27.25. The best multiple shoot formation of 97.95% was observed on the medium M11 (MS+1.14 μ M IAA+19.97 μ M BAP) which was at par to 95.71% on medium M14 (B5 + 1.14 μ M IAA+ 19.97 μ M BAP). The medium M4 (MS + 22.19 μ M BAP) showed the next best frequency of shoot proliferation (93.59%). The culture on medium M11 resulted in the highest number of differentiated shoots per culture (27.25) which was at par to medium M14 (25.50) and closely followed by medium M4 (23.25). The other media M3 (MS + 17.75 μ M BAP) and M5 (MS + 23.23 μ M KIN) also resulted in good multiple shoot formation (Table 1). Whereas, the media M6 (MS + 4.54 μ M TDZ) and M7 (MS + 9.08 μ M TDZ) were at par for the induction of low shoot multiplication.

The multiplied shoots on medium M11 were further subcultured to increase the total number of *in vitro* developed shoots onto the same medium (Figure 1C). The multiple shoot formation frequency from subcultured *in vitro* developed shoots was not affected by the number of culture cycles generally and remained cent-percent. However, number of differentiated multiple shoots per subculture varied with the increasing culture cycles. The mean number of differentiated shoots per culture was at par till 3rd subculture. Furthermore, it remained more or less same upto 5th subculture cycle. The potential of subcultured propagule multiplication in terms of number of differentiated shoots per culture declined by 16.67% in 6th subculture, 28.07% in 7th subculture and 40.35% in 8th subculture

(Table 2). The differentiated multiple shoots were divided into single shoots and subcultured for the proper development of roots on the medium MS + 4.92 μ M IBA following the work of Kumari and Kumar (2016) (Figure 1D). The observed root formation from *in vitro* developed plantlets was cent-percent on this medium.

The tissue culture derived plantlets of banana cv. Robusta comprising well developed roots and healthy shoot were acclimatized progressively to natural environment was apposite in term of establishment to field conditions, plant growth and morphology in contrast to *ex vitro* developed parent plant (Figures 1 E, F).

Shoot apices although are not readily available explants due to less sprouting of suckers from the base of banana in *ex vitro* conditions, but they are the best explants to acquire multiple propagules with less variations in *in vitro* conditions. Multiple shoot formation was supported by the media having moderate to high concentrations of only cytokinin and low auxin with high cytokinin. The best multiple shoot formation and the highest number of differentiated shoots was observed on medium M11, which was at par to medium M14. Both of the media are constituted with low concentrations of auxin IAA and high concentrations of cytokinin BAP. The classical hypothesis of 'chemical control of organogenesis' suggested that low concentrations of auxin with high concentrations of cytokinin supported multiple shoot formation (Skoog and Miller, 1957). The concentration of auxin in cells determines the position of quiescent centre (Jiang *et al.*, 2003). The threshold concentration of auxin in the nucleus resulted in degradation of AUX/IAA a transcriptional repressor and subsequent activation of transcription of auxin biosynthesis pathway by dimerization of ARFs (Auxin response factors) proteins (Taiz and Zeiger, 2010).

Table.1 The effect of different media on percentage multiple shoot formation and number of differentiated shoots per cultured shoot apex of banana cv. Robusta

S. No.	Media Name	Media Compositions	Multiple shoot formation % (mean ± SE)	No. of shoot/explants
1	M1	MS + 4.44 µM BAP	62.02 ^e ± 1.52	14.00 ^{cd} ± 0.41
2	M2	MS + 8.88 µM BAP	63.04 ^e ± 1.34	16.50 ^{bc} ± 0.50
3	M3	MS + 17.75 µM BAP	72.46 ^c ± 0.67	17.25 ^b ± 0.85
4	M4	MS + 22.19 µM BAP	93.59 ^b ± 1.44	23.25 ± 0.48
5	M5	MS + 23.23 µM KIN	71.28 ^{cd} ± 0.67	16.50 ^{bc} ± 0.64
6	M6	MS + 4.54 µM TDZ	26.43 ± 1.83	7.25 ^f ± 0.48
7	M7	MS + 9.08 µM TDZ	33.88 ± 0.94	8.25 ^f ± 0.48
8	M8	MS + 9.05 µM 2,4-D + 19.97 µM BAP	42.29 ^f ± 0.84	12.00 ^d ± 0.91
9	M9	MS + 9.05 µM 2,4-D + 18.59 µM KIN	43.74 ^f ± 2.55	10.00 ^e ± 0.71
10	M10	MS + 0.2 µM IAA + 4.5 µM BAP	67.27 ^d ± 2.85	12.75 ^d ± 0.85
11	M11	MS + 1.14 µM IAA + 19.97 µM BAP	97.95 ^a ± 1.19	27.25 ^a ± 0.48
12	M12	B5 + 4.44 µM BAP	40.34 ^f ± 0.91	11.25 ^{de} ± 0.48
13	M13	B5 + 8.88 µM BAP	62.40 ^c ± 1.08	15.25 ^c ± 0.85
14	M14	B5 + 1.14 µM IAA + 19.97 µM BAP	95.71 ^{ab} ± 0.22	25.50 ^a ± 0.64
15	M15	MS + 4.65 µM KIN + 6.65 µM BAP + 10mg Bavistin	73.16 ^c ± 2.30	18.25 ^b ± 0.63
16	M16	Modified MS2* + 1.14 µM IAA + 19.97 µM BAP	82.54 ± 0.91	21.00 ± 0.91
		Mean	64.26	16.02
		SE(m)	1.51	0.67
		CD	4.29	1.91
		CV	4.68	8.35

Each medium was supplemented with 3% sucrose as carbon source and solidified with 0.8% agar. *Modified MS2 (without NH₄NO₃, KNO₃-2020 mg/l, and decreased concentration of CaCl₂·2H₂O- 220.50 mg/l, KH₂PO₄ – 44 mg/l and H₃BO₃ – 1.25 mg/l). Values expressed as mean ± SE. Mean value (n=4) in columns bearing same letter are not significantly different using Duncan's Multiple Range Test at 5% level.

Figure.1 A: The cultured shoot apex; B: Differentiated shoots on medium M 11 (MS + 1.14 µM IAA + 19.97 µM BAP); C: Multiplication of shoots after subculture on medium M 11; D: Rhizogenesis from *in vitro* developed shoot on medium MS + 4.92 µM IBA; E: Acclimatization of plantlets and F: Pot transfer of acclimatized plant.

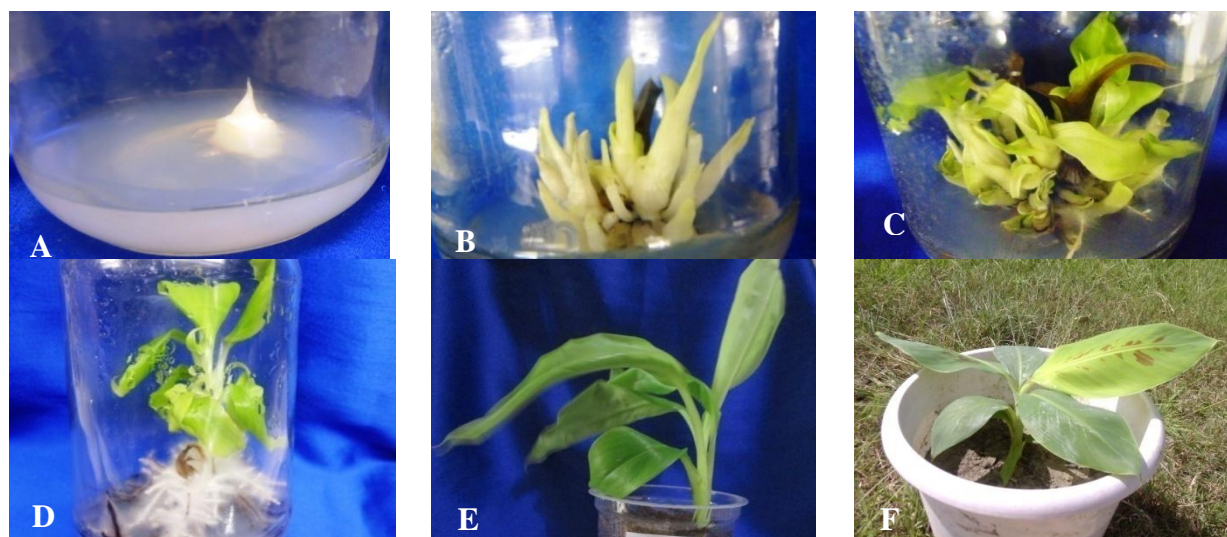


Table.2 The effect of subculture cycle on number of shoot differentiation per culture

S. No.	Media	Number of multiple shoots/subculture	Percentage change in number of multiple shoots/subculture
1	Sub1	28.50 ^a ± 0.64	0
2	Sub2	28.75 ^a ± 0.48	+ 0.88
3	Sub3	27.25 ^{ab} ± 0.48	- 4.39
4	Sub4	26.75 ^b ± 0.48	- 6.14
5	Sub5	26.75 ^b ± 0.63	- 6.14
6	Sub6	23.75 ± 0.85	- 16.67
7	Sub7	20.50 ± 0.64	- 28.07
8	Sub8	17.00 ± 0.71	- 40.35
	Mean	24.91	
	SE(m)	0.63	
	CD	1.84	
	CV	5.04	

Values expressed as mean ± SE. Mean value (n=4) in columns bearing same letter are not significantly different using Duncan's Multiple Range Test at 5% level.

Cytokinin codes for the repressor protein AUX/IAA, which in turn inhibits the biosynthesis of auxin in cells (Dello Ioio *et al.*, 2008). An optimal concentration of plant growth hormone cytokinin in medium helps in cell division and multiplication. Supplementation of auxin in low concentration exogenously in medium with high concentration of cytokinin helps plant (explants or shoot apices) to maintain a level of the hormone which is necessary for the maintenance of undifferentiated SAM descendents in cell. These hormonal signals lend a hand to distinct genes and gene combinations as *SCARECROW (SCR)* and *SHORT-ROOT (SHR)* to specify root and shoot meristem formation (Gilbert, 2010), which in turn enhances the shoot differentiation.

The media M11 and M14 which resulted in the highest frequency and number of differentiated shoots per culture had lower auxin and higher cytokinin concentrations. Similar results of the maximum shoot differentiation from the cultured shoot apices of different cultivars of banana on media with

similar proportion of auxin and cytokinin was observed by Iqbal, *et al.*, (2013) and Ahmed, *et al.*, (2014). However, Iqbal *et al.*, (2013) utilized the medium MS + 22.2 µM BAP and 8.56 µM IAA with higher concentrations of same auxin and cytokinin along with 10% coconut water for shoot multiplication from shoot tip culture of cv. Williams. Ahmed, *et al.*, (2014) used medium (MS + 17.75 µM BAP + 11.42 µM IAA) with low concentration of BAP but higher concentration of IAA compared to present work for development of adventitious shoots from shoot tip culture of cv. Grand Naine. Both the workers worked on autotriploid (AAA) cultivars of banana like cv. Robusta of present study. Choudhary, *et al.*, (2014) reported the maximum 15.6 numbers of shoots per culture on MS medium with 2.0 mg/l BAP and 0.5 mg/l NAA, and 9.7 per culture on MS medium supplemented with 30 mg/l adenine sulphate, 2 mg/l BAP and 0.5 mg/l NAA from the same cv. Robusta. The next best medium for multiple shoot differentiation was M4 (MS + 22.19 µM BAP). Similarly, Arinaitwe, *et al.*, (2000) got the best shoot proliferation on higher

concentration of only BAP (28.8 μM). They showed that BAP was the best cytokinin for shoot proliferation but the shoot proliferation rate of 3.5 and 8.0 per culture in two cultivars Kibuzi and Bwara respectively with same genome as of cv. Robusta was much less than observed in the present work.

The impact of media M6 (MS + 4.54 μM TDZ) and M7 (MS + 9.08 μM TDZ) on induction of shoot multiplication were at par. TDZ is a diphenyl urea derived *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea resistant to all cytokinin oxidases and induces the accumulation of endogenous cytokinins (Kaminek, 1992). Arinaitwe, *et al.*, (2000) studied the effect of TDZ for the first time on proliferation rate in *Musa* spp. They observed that the proliferation rates of cvs. Bwara and Kibuzi increased with increasing concentrations of TDZ (0.045 μM to 1.14 μM) in media and afterwards decreased with increased concentrations of TDZ (6.81 μM) as found in the present work. Lee (2005) also reported that lower concentration of TDZ (0.2 mg/l) was more effective than higher concentrations. Similar result of multiple shoot formation response on TDZ was reported by Kumari and Kumar (2016).

The scope of an efficient micropropagation system is to achieve a very high propagule multiplication rate. The shoot apices culture of banana cultivar Robusta resulted in establishment of high rate of multiplication of shoots on medium M11. The frequency of multiple shoot differentiation was not affected generally, but the number of differentiated multiple shoots per subcultured *in vitro* developed shoots varied with the increasing culture cycles. The mean number of differentiated shoots per culture remained more or less the same upto 5th subculture cycle and thereafter encumbered with further subcultures. Similar observation of subculturing effect on the proliferation rate of shoot tip culture of banana was reported by

Kumar, *et al.*, (2005) and Kumari and Kumar (2016). Contrary to present work Kumar, *et al.*, (2005) got higher number of differentiated shoots in 3rd to 5th subcultures.

The stressful environment of plant tissue culture imposed induction and accumulation of variation in cultured plant tissues. Multiple *in vitro* tissue culture factors induced variations in plant phenotype, gene expression and genotypic features. The observed decline in multiple shoot differentiation efficiency of subcultured *in vitro* developed shoots after 5th subculture might be due to the accumulation of variations resulting from somaclonal variations which prevented regeneration of shoots from variant cells. Similarly, contribution of subculturing onto multiplication ability of *in vitro* developed shoot buds were recognized by Us-Camas, *et al.*, (2014) and Saraswathi, *et al.*, (2014). Aremu, *et al.*, (2013) also reported significant effect of subculturing onto proliferation and multiplication potential of banana cv. 'Williams' after 6th subculture as found in present investigation. Many investigators reported the impact of subculture cycles on the efficiency of differentiation of multiple shoots was influenced by the genotype of the banana cultivars (Abdullah *et al.*, 1997; Rodrigues *et al.*, 1998).

Development of roots or rhizogenesis is an important organogenesis without which *in vitro* developed shoots may not be converted in to plants and transplanted to *ex vitro* conditions. The *in vitro* multiplied plant propagaules were divided into single shoots and inoculated onto root inducing medium consisting of 4.92 μM IBA. Auxin induces vascular differentiation and plays an important role in induction and development of roots (North *et al.*, 2012; Ngomuo *et al.*, 2014). Indole-3-butyric acid (IBA), a persuasive plant auxin, was also observed to produce the greatest number of roots in present experiment. Similarly, Rahman, *et al.*,

(2013) and Saraswathi, *et al.*, (2014) reported its effectiveness in development of roots in banana cultivars. Manchanda, *et al.*, (2012) and Kumari and Kumar (2016) obtained profuse rooting from the base of cultured shoot of banana cultivars utilizing similar concentration of IBA (1.0 mg/l). Strosses, *et al.*, (2004) also subcultured *in vitro* developed shoots on the same medium for rhizogenesis and got cent-percent response during regeneration of banana plantlets.

The well rooted tissue culture plantlets of cv. Robusta consisting of healthy shoot were acclimatized progressively to *ex vitro* environmental conditions resulted in successful establishment of these plants at fields. Thus, the work resulted in accomplishment of more efficient and robust micropropagation system with a high multiplication rate for the valuable cv. Robusta as compared to earlier results (Senthilkumar and Ramsundar, 2009; Choudhary *et al.*, 2014).

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