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RESEARCH ARTICLE

HIGH FREQUENCY REGENERATION FROM THE LEAF CALLUS OF *MUSA PARADISIACA CV.* 'PUTTABALE' (AB GENOME) IN MALNAD REGION OF THE WESTERN GHATS

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ABSTRACT

Indirect organogenesis protocol was developed for high frequency shoot induction from leaf sheath disc callus of *Musa paradisiaca cv.* Puttabale (AB genome) an indigenous banana variety cultivated in Malnad region of the Western Ghats, Karnataka, India, which is known for its delicious taste and flavor but highly prone to disease and pests. The best callogenic frequency (97.5%) was observed on Murashige and Skoog (MS) basal nutrients augmented with 160 mg/l Adenine sulfate, 100 mg/l Tyrosine and growth regulators at the concentration of 4 mg/l 2, 4 Dichlorophenyloxy acetic acid (2, 4-D) and 1 mg/l 6-Furfurylaminopurine (Kn). High frequency of shoot bud organogenesis (22.80 ± 2.57) was observed at the concentration of 0.3 mg/l Thidiazuron (TDZ) and 4 mg/l 6-Benzylaminopurine (BAP). The multiple shoots developed into rooted plantlets on MS media fortified with 0.5 mg/l Naphthalene acetic acid (NAA) and 0.2% activated charcoal. The leaf calli regenerants were successfully acclimatized in the greenhouse and transformed to the farmland. Morphoagronomic evaluation of the regenerants in the farmyard showed elite characteristics in plant height, pseudostem perimeter, number of hands, number of total fingers and total weight of bunches than the *in vivo* plants.

INTRODUCTION

Musa paradisiaca cv. Puttabale (AB group) is an indigenous banana variety commonly cultivated in the Malnad region of Karnataka, India. The fruits are valued for its delicious taste and flavor but highly prone to disease and pests such as, Fusarium wilt (*Fusarium oxysporum f.sp. cubense*), black Sigatoka (*Mycosphaerella fugiensis*), viruses (Banana bunchy top viruses) and nematodes (*Radopholus similes*) (Sasson, 1997). Application of high doses of fungicides and pesticides for the eradication of pathogens causes serious consequences for the environment. The improvement of this species by traditional breeding methods has not been very successful (Stover and Buddenhagen, 1986), due to its polyploidy nature, heterozygosity, sterility, low fertility and limited genetic variability (Simmonds, 1976). Hence, mass propagation of selected genotypes and somaclonal variation for banana improvement are based on reliable plant regeneration techniques to meet the increasing need for seedlings. To date much research work has been successfully carried out in tissue culture protocol on a wide range of banana cultivars using apical meristem (Mante and Tepper, 1983), shoot-tip (Kanchanapoom and Chanadang, 2001; Buah *et al.,* 2010), floral explants (Cronauer and Krikorian, 1985; Cote *et al.,* 1996; Ganapathi *et al.,* 1999; Gomez *et al.,* 2001), protoplast culture (Panis *et al.,* 1993), embryos culture (Escalant *et al.,* 1989), organ formation (Jarret *et al.,* 1985) and immature fruits as a explants (John Nelson Buah, *et al.,* 2000; Msogoya, 2006). Literature survey also revealed that in many species and cultivars of banana protocols are standardized and commercialized for clonal multiplication from the apical meristem (Rout *et al.,* 2000). But the reports are very few on the regeneration of plantlets through the leaf callus culture (Okole and Schulz, 1996). Further, the protocol for mass multiplication plantlets for this indigenous banana *cv.* Puttabale has not yet been standardized so far. In this study, we have investigated the high frequency shoot induction from leaf callus and evaluation of morpho agronomic characters of the calli regenerants of banana *cv*. Puttabale in the farmyard.

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MATERIALS AND METHODS

Preparation of explants

Healthy and elite side-suckers of *Musa paradisiaca cv.* puttabale were collected from banana farmyards in Malnad region (Shimoga district), Karnataka, India. The suckers were thoroughly washed with tap water and shoot-tip explants prepared by removing the outer leafy sheaths of suckers with a clean knife. Tissue blocks containing shoot-tips and rhizomatous bases were surface disinfected for 5 min in 70% ethanol followed by immersion in 1% sodium hypochlorite for 10 min, both solutions containing a few drops of Tween 20, then rinsed thrice in sterile distilled water and excised of few

sheathing leaves and part of corm tissue. Aseptic shoots-tips (~3-cm height) were cultured on MS (16 Murashige and Skoog, 1962) medium supplemented with 3 mg/l BAP, 0.2 mg/l IAA, 100 mg/l Tyrosine, 160 mg/l Adenine sulfate. The leaf sheath disc or lamina of the *in vitro* grown shoots were aseptically excised and inoculated onto the callus induction medium.

Callus induction

For callus induction, 1cm the leaf sheath discs were aseptically excised in the laminar hood and washed in the sterilized solution of 0.02% ascorbic acid for 5 min, rinsed with the sterilised distilled water, blotted and then inoculated by placing abaxial surface of the inoculum in contact with medium The callogenic media consisted of MS basal nutrients augmented with 30 g/l sucrose, 8 g/l agar, 160 mg/l adenine sulfate and 100 mg/l tyrosine. The plant growth regulators 2, 4-D and Kn were tested at the range of 2 mg/l to 5 mg/l and 0.5 mg/l to 2 mg/l, respectively. The cultures were incubated at 25 ± 2ºC with 16 hrs photoperiod and callogenic frequency of the leaf sheath discs were evaluated after 45 days of inoculation using ten replications for each hormonal concentration.

Regeneration of plantlets

The leaf sheath disc calli were sub-cultured onto the shoot differentiating media containing MS basal nutrients with 100 mg/l adenine sulfate, 100 mg/l tyrosine .The combined effect of cytokinines BAP and TDZ were tested at the range of BAP 2 mg/l to 6 mg/l and TDZ 0.2 mg/l – 0.4 mg/l, respectively. The mean number of multiple shoots differentiated per callus mass was evaluated after 45 days of inoculation of leaf calli on to the differentiating media supplemented with different combinations of BAP and TDZ. The data was statistically evaluated by using ezANOVA tool (0.98 versions). For rhizogenesis nodular mass containing multiple shoots were aseptically transferred to maturation medium augmented with 0.5 mg/l NAA, 0.2% activated charcoal, 3% sucrose and 0.8% agar-agar and maintained in the culture condition for a period of 4 weeks.

Evaluation of morphoagronomic characters of the regenerants

The rooted shoots were washed with sterile distilled water and transferred to plastic pots with sterile cocopeat:perlite (80:20 v/v) (Durga Industries, India). The plantlets were maintained in polythene house at 70% relative humidity and 28 ± 2 ^{0}C for 2 weeks, and subsequently transferred to secondary hardening to polythene bags filled with garden soil, sand, and cattle dung manure in the ratio 1:1:2 in a greenhouse (relative humidity 80%, 34 ± 2 ^{0}C) for one month before planting in the field and survival rates was recorded. The plants were planted at a distance of five feet each. After eight months of cultivation five plants were randomly selected for morphoagronomic evaluation. The characters such as, height of the plant, pseudostem perimeter, number of suckers and number of leaves at the flowering moment, total weight of bunches, number of hands and number of total fingers were evaluated by comparing with the *in-vivo* grown plant. The experiments were carried out at farm field.

RESULTS AND DISCUSSION

Callus induction from leaf sheath disc explants

Callus induction was noticed from the excised margin of leaf discs after 30 -35 days of incubation on MS medium augmented with 100 mg/l Tyrosine, 160 mg/l Adenine sulfate and growth regulators 2, 4-D (2 mg/l to 5 mg/l) and Kn (0.5 mg/l to 2.0 mg/l). Frequency of callus formation was highest (97.50%) at the concentration of 4 mg/l 2, 4-D and 1 mg/l Kn (Table 1). Phenolic exudation is the major problem in banana *cv.* puttabale leaf disc culture for callus induction and maintenance. The browning of the media has been avoided by washing of the leaf disc inoculum with the antioxidant 0.02% ascorbic acid for 5 min prior to inoculation and incorporate on of 100 mg/l Tyrosine into the media. In the meristem culture of banana *cv.* Desi of Pakistan (Malik *et al.,* 2000) and floral bud culture of banana *cv.* Kanthali of Bangladesh (Sarder Nasir Uddin and Soubir Titov, 2007) also reported the pre-treatment with the antioxidants and incorporation of 100 mg/l Tyrosine into the media to avoid exudation of phenolics. In the present investigation frequent subculturing of the calli at a 3-week interval could minimize the accumulation of phenolics and increase the rate of callus proliferation. At optimal concentration between 25 to 30 days of culture the leaf sheath disc inoculum became swollen and enlarged in to ten folds of its normal size, gradually initiation of flashy callus noticed from the excised margin of the explant (Fig. 1A). Upon subculturing onto the same media callogenesis was proceeded all over the surface of the explants and appeared in the form of yellowish nodular mass. However, no sign of differentiation was observed even after five successive subculturing of the calli onto the same media

Multiple shoot differentiation from callus

The interaction of exogenously supplemented cytokinins, BAP at higher levels (2 mg/l to 6 mg/l) and TDZ at lower levels (0.2 mg/l to 0.4 mg/l) provoked multiple shoot differentiation from the calli. The mean number of shoots organized per callus mass at each combination of BAP and TDZ is shown in the Table 2. When these growth regulators were tested individually at different concentrations, only proliferation of the nodular calli with photosynthetic loci was noticed. In the clonal multiplication of banana cultivar, BAP alone at different levels was tested to induce shoot multiplication from the shoot tips (Malik *et al.,* 2000). In the present investigation BAP alone at the range of (2 mg/l to 6 mg/l) could not induce the shoot differentiating potency of the leaf calli. Similarly, the protocol for *in vitro* propagation of banana (Musa spp.) using thidiazuron from shoot tip was optimized (Hamide and Mustafa, 2006). On the contrary, interaction with the TDZ at lower concentration (0.2 to 0.4 mg/l) would provoke multiple shoot differentiation from the leaf calli. The shoot multiplication was optimized at the concentration of 4 mg/l BAP and 0.3 mg/l TDZ with a mean of 22.80 ± 2.57 shoots per callus. On differentiating media, the yellowish nodular mass turned in to whitish nodules (Fig. 1B), which showed the resemblance of somatic embryos but the sequential stages of the somatic embryos and the appearance of the embryonic root *primordium* were not traced out. On further incubation formation of photosynthetic loci appeared from the tip of the nodules and these photosynthetic loci were transformed in to shoot buds. In a 40 days old culture the shoot buds grew up

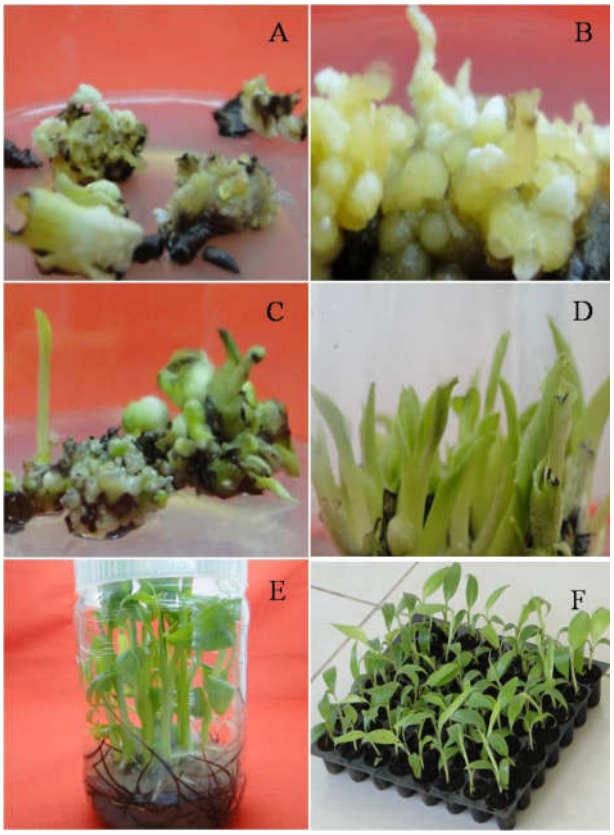

Figure 1: High frequency regeneration from the leaf callus *Musa paradisiaca cv.* 'Puttabale'

A. Callus initiation from the leaf inoculum MS+ 4 mg/l 2, 4-D +1 mg/l Kn.
B. Organization of embryo like nodular callus on MS+ 4 mg/l BAP +0.3 mg/l TDZ
C. Sprouting of multiple shoot buds from the leaf callus on MS+ 4 mg/l BAP +0.3 mg/l TDZ
D. Growth of multiple shoots on differentiation media.
E. Root induction and complete plantlet development on MS+ 0.5 mg/l NAA+ 0.2% activated charcoal
F. Early stage of Primary hardening of regenerants in cocopeat.

Figure 2: High frequency regeneration from the leaf callus *Musa paradisiaca cv.* 'Puttabale'.

A. Primary hardening of regenerants in cocopeat.
B. Secondary hardening of regenerants in Polythene bag.
C. Calli derived regenerants in field condition.
D. Yield of calli derived regenerants in field condition.

well with two to three leaf primordia (Fig. 3C). A piece of differentiating callus when isolated aseptically and subcultured on to the same concentration medium revealed further multiplication of shoot buds (Fig. 1D) and the differentiating potency was maintained up to 8 successive subcultures, later processes of elongation of shoot buds was decreased. It was known that genetic variability can be induced through indirect organogenesis. In plantain, banana cultivar protocol was standardized and commercialized for mass multiplication through clonal propagation and the investigator (Arias, 1993; Gowen, 1995) does not given much importance for callogenesis and organogenesis through the calli. But callus regeneration is also a boon for the induction of somoclonal variant. The earlier investigators (Okole and Schulz, 1996) were succeeded to induce shoot bud organogenesis from the leaf disc calli of plantain cultivar *Grand naine*. In the present investigation, high frequency shoot induction was achieved from the leaf sheath calli of *Musa paradisiaca cv.* Puttabale.

Rhizogenesis from the microshoots was achieved on MS medium fortified with 0.25 to 0.75 mg/l NAA. The individuals shoot buds were harvested from the clump when they attained a length of 4-5 cm with 3 - 4 leaf. They were transferred to MS medium containing 0.5 mg/l NAA + 0.2% activated charcoal. Roots were initiated from the base of microshoots after three weeks of culture (Fig. 1E). A similar result obtained (Berg and Bustamante, 1974) with 1.0 mg/l NAA in the culture medium shows better rooting than indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA). The well rooted plantlets were hardened primarily in cocopeats, maintained in polythene house for a period of two weeks (Fig. 1F, 2A). Then transferred to green house for secondary hardening in polythene bags containing garden soil, sand and cattle dung manure in the ratio 1:1:2 (Fig. 2B). The regenerants grew the height of 10 to 15 cm with three to four lustor greenish leaves was transferred to the field condition.

Evaluation of morphoagronomic characters

Morphoagronomical features of five leaf calli regenerants were comparatively evaluated with the *in vivo* plants and the data is depicted in the Table 3. After eight months of acclimatization of *in-vitro* regenerants the plant height, pseudostem perimeter, leaves at flowering time, number of hands, number of total fingers and total weight of bunches (Fig. 2B, 2C) were more as compared to *in vivo* plants. In addition the number of suckers were also more in *in vitro* regenrants. This is the main problem noticed in the cultivation of tissue cultured banana plantlets and has to be removed for better yield. Several investigators reported the superior growth and yield of the *in vitro* regenerants compared to *in vivo* plants (Robinson, *et al.,* 1993; John Nelson Buah, *et al.,* 2000; Msogoya, 2006; Shaileas Vasane, *et al.,* 2010). In the

Table 1. Effect of 2,4-D and Kn on the frequency of callus formation from leaf discs of *M. paradiriaca cv*. Puttabale

Growth regulators		Frequency of callus formation %
2,4-D mg/l	Kn mg/l	
2	0.0	27.5
2	0.5	22.5
2	1.0	17.5
2	1.5	00.0
2	2.0	00.0
3	0.0	35.5
3	0.5	42.5
3	1.0	67.5
3	1.5	30.0
3	2.0	00.0
4	0.0	50.0
4	0.5	57.5
4	1.0	97.5
4	1.5	42.5
4	2.0	07.5
5	0.0	27.5
5	0.5	62.5
5	1.0	37.5
5	1.5	10.0
5	2.0	00.0

The value of each combination consisted of percentage of callus induction from the four leaf sheath discs of 4 × 10 replicates.

Table 2. Effect of BAP and TDZ on shoot bud differentiation from the leaf disc calli of *M. paradiriaca cv.* Puttabale

Plant growth regulators mg/l		Number of shoot buds/ callus Mean ± SD
BAP	TDZ	
2	0.2	7.00 ± 1.56
2	0.3	7.50 ± 1.43
2	0.4	10.50 ± 1.72
3	0.2	8.50 ± 1.58
3	0.3	10.20 ± 2.25
3	0.4	9.30 ± 2.21
4	0.2	14.20 ± 3.79
4	0.3	22.80 ± 2.57
4	0.4	17.30 ± 2.21
5	0.2	6.70 ± 1.57
5	0.3	9.10 ± 2.92
5	0.4	3.60 ± 1.17
6	0.2	2.60 ± 2.22
6	0.3	2.30 ± 1.34
6	0.4	3.30 ± 1.49
F valve		71.7

The value of each combination consisted of mean ± S.D. of 10 replicates.
The F-value is significantly different when $p < 0.05$.

Table 3: Morphoagronomic characteristics of the leaf disc calli regenerants in banana farm yard

Banana plants in field	Height plants (m)	Pseudostem circumference (cm)	No. of Suckers	Leaves at flowering moment	No. of Hands	No. of total Fingers	Total Weight of Bunches (kg)
Leaf calli regenerants	5.60±0.79	54.32±2.70	10.40±1.14	13.80±0.84	11.20±1.30	145.60±15.96	15.50±0.94
In vivo grown	5.34±0.76	52.16±1.24	5.60±1.14	10.60±1.14	10.40±1.14	120.00±7.11	13.02±1.97

The value of each concentration consisted of mean ± S.D of 5 replicates.

present study also the regenerants exhibited elite characteristics as compared to *in vivo* plants. Further work will be in progress on the genomic analysis for genetic variation of the *in vitro* leaf calli regenerants. In conclusion, for *Musa paradisiaca cv.* 'puttabale' (AB group) known for its delicious taste. An efficient protocol has been standardized for the Western Ghats banana for mass multiplication through leaf sheath disc calli and reintroduction of plantlets to the farm yard.

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