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Spermine-Induced Morphogenesis and Effect of Partial Immersion System on the Shoot Cultures of Banana

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Abstract Contribution of exogenous polyamines (PAs) and polyamine-inhibitors on plantlet regeneration patterns of banana (*cv.* Nanjanagudu Rasabale-AAB) was studied and the performance of regenerated shoots in temporary immersion system was evaluated. The rhizome explants (without shoot bud) of in vitro shoots produced a mixture of embryogenic and nonembryogenic calli on modified MS medium. The analyses of endogenous pools of polyamines showed higher levels of PAs in embryogenic than in nonembryogenic calli. Supplementation of various levels of (10–50 μM) spermine (Spm), spermidine (Spd), and putrescine (Put) to cultures with secondary embryogenesis showed that about 50% of embryogenic calli rapidly produced secondary embryos only in the presence 40 μM Spm but not in other treatments. The crucial role of Spm was further confirmed by the use of 0.1 mM each of α-DL-Difluromethylornithine and α-DL-Difluromethylarginine along with Spm where the presence of inhibitors concomitantly inhibited the secondary embryogenesis. The shoots obtained from the embryogenic cultures were checked for their performance on solid medium (SM) and partial immersion system (PIS). The rate of shoot multiplication was higher in PIS than in SM throughout 6 weeks culture period. Uniformity in elongation of all the shoot buds was observed in PIS but not in SM. Evaluation for the acclimatization, survival under greenhouse conditions revealed the better performance of PIS-derived plants than those from SM.

Keywords Embryogenesis · *Musa* · Partial immersion system · Polyamine

Abbreviations

2iP	N^6-(2-isopentenyl)adenine
BA	Benzyladenine
DFMA	α-DL-Difluromethylarginine
DFMO	α-DL-Difluromethylornithine
GA	Gibberellic acid
HPLC	High performance liquid chromatography
PAs	Polyamines

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PIS Partial Immersion System
Put Putrescine
SM Solid Medium
Spd Spermidine
Spm Spermine

Introduction

Edible banana cultivars are sterile triploids or tetraploids, and they are normally propagated by vegetative means. The progress made over the last decades in in vitro-based technologies has contributed immensely for the production of high quality planting material of desired clones. Propagation of banana through in vitro techniques has been reported by several workers using different explant sources as well as regeneration pathways. Diverse procedures have been originally described for somatic embryo induction in banana, differing from each other mainly due to the genotypic differences and use of various types of explants such as zygotic embryos [1], foliar bases and corm slices [2], in vitro-cultured meristems [3–6], and immature male [7, 8] and female [9] flowers.

A variety of exogenous growth regulators have been reported useful for banana micropropagation [3, 6, 10, 11]. This apart, banana shoots in vitro are known to synthesize and accumulate a natural cytokinin, 2iP at the basal portions of shoot clusters resulting in de novo bud formation, especially under an exogenous supply of benzyladenine (BA) [12, 13].

Another class of molecules, namely polyamines (PAs), such as Putrescine (Put), Spermidine (Spd), and Spermine (Spm), the chemically nonprotein straight-chain aliphatic amines, are known to play important role in various cellular processes such as DNA replication, cell division, protein synthesis, responses to abiotic stress, rhizogenesis, flower development, and in vitro flower induction [14, 15]. PAs have also been found implicated in morphogenic process where there are increasing evidences from several studies indicating that they play a crucial role either directly in somatic embryogenesis and regeneration or indirectly through the release of nitric oxide or inhibition of ethylene biosynthesis [14–17].

For in vitro propagation of plants, partial immersion bioreactor systems (PIS) have shown higher advantages compared to semisolid and liquid medium, in terms of faster multiplication or elongation of shoots. Such improved results were linked to the situation offered by PIS where the latter combines the advantages of both gelled and liquid medium. These advantages are due to intermittent aeration and higher availability of nutrients in liquid. A low-cost Growtek™ bioreactor has been designed and commercialized with unique features like floating and rotating ex-plant holder with perforated ex-plant support and a side tube for medium changing, culture feeding, and content monitoring. The usefulness of Growtek™ in terms of enhanced multiplication rates, reduced bioreactor costs, saving in incubation time, the minimization of contamination, and plantlet transfer without root injury has been well documented [18]. Therefore, a set of experiments were designed combining the effects of chemical parameters such as growth regulators and PAs on shoot or embryogenic cultures of banana under normal conditions using solid and liquid medium and compared with the similarly cultured shoots in Growtek™ bioreactors. The effects of these treatments were basically analyzed by checking the morphogenic events of organization of pro-embryogenic masses and further formation of shoot cultures and their performances until they were transferred to soil. In addition, at different stages of experiments, the effects of exogenous PAs were also monitored by analyzing the pools of endogenous PAs. To our knowledge, this is the first report in banana on the use of PAs for

the propagation where involvements of spermine in enhancing the rate of shoot multiplication and plantlet regeneration from somatic embryos have been documented.

Materials and Methods

Plant Material

The rhizomes of sword suckers of an endangered dessert banana *cv.* Nanjanagudu Rasabale (AAB) were used to excise the meristem and establish shoot cultures as described earlier [11]. The mother plant was maintained at the backyard of authors' department.

Culture Medium and Incubation Conditions

The aseptic shoot cultures used in the present study were established as reported earlier [11] and were maintained on M1 medium based on strength of MS basal salt mixtures [19] with additional 1,000.0 mg l^{-1} KNO_3 and 1.0 ml l^{-1} vitamin mixture [2] supplemented with 100 mg l^{-1} ascorbic acid, 1.0 mg l^{-1} BA, 0.2 mg l^{-1} IBA, 30 g l^{-1} sucrose, and 2.5 g l^{-1} gelrite (Sigma, St. Louis, USA). The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Cultures were maintained at 25±1 °C under a 16-h photoperiod (approximately 320 μmol m^{-2} s^{-1} PFD) provided by incandescent lamps (Philips, New Delhi, India). The in vitro multiple shoot cultures were established and maintained on M2 medium, designed based on MS medium supplemented with 1,238 mg l^{-1} NH_4NO_3, 2.0 mg l^{-1}BA, 1.0 mg l^{-1} Kinetin, 80 mg l^{-1} adenine sulphate, 25 g l^{-1} sucrose, and 2.5 g l^{-1} gelrite. The shoots and the rhizome explants obtained from these cultures were used subsequently for embryogenesis, and callusy rhizome formations were induced as reported earlier [13]. For plantlet formation and shoot elongation, M2 medium was used.

Polyamine Treatment

Concentrations of 10,20,30,40, and 50 μM of each PA viz., Spm, Spd, and Put (Sigma, St. Louis, USA) were tested by adding to control (M2) medium**.** These concentrations were selected based on an earlier study of Laine et al [20]. Culture segments, of uniform size (∅= 0.5 cm) having embryogenic mass, rhizome with callus and shoot clusters with shoot buds varying in length from 0.2–0.5 cm were selected and cultured in M2 with or without PAs. For each treatment, five segments were transferred to each bottle (10 cm in height and 6 cm in diameter) ×5. The observations on growth and morphological changes were recorded periodically; after which, the cultures were transferred to fresh respective medium by vertically splitting the culture into three pieces. Three such successive subcultures were made; after which, the final morphological observations and endogenous levels of PAs were recorded. Inhibitors of PA biosynthesis (DFMA and DFMO, Sigma, St. Louis, USA) were added (membrane-filtered) to the control medium M2 and to PA-containing medium at concentrations ranging from 0.1 to 0.5 mM. Two independent experiments were performed, using at least five replicate samples in each; the data obtained were averaged.

Extraction and Analysis of Free PAs

The extraction of PAs and high performance liquid chromatography (HPLC) analyses were conducted according to the method of Flores and Galston [21] and authentic standards of

Put, Spd, and Spm (Sigma, St. Louis, USA) were benzoylated following the procedure described by Flores and Galston [21]. Free PAs were extracted by homogenizing the plant materials from two culture samples (each 100 mg of tissue) in 1 ml of 5% ice-cold perchloric acid using a pestle and mortar. The homogenate was then centrifuged for 30 min at 20,000×*g*. Free PAs in the supernatant were benzoylated as Flores and Galston [21] and determined using an HPLC (Shimadzu LC6A, Tokyo, Japan). The elution system consisted of MeOH/H_2O (64:36) solvent, running isocratically with a flow rate of 1.0 ml/min. The benzoylated PAs were eluted through a C_{18} column (300×4.6 mm i.d., with pore size of 5 μm), an SLC-6A system controller, and a CR4A data processor was used. Compound detection was done through a ultraviolet detector SPD-AV set at a sensitivity of 0.04 AUFC and absorbance at 254 nm. A relative calibration procedure was used to determine the PAs in the samples, using standard of Put, Spd, and Spm. Peak areas and retention times were measured by comparing with standard PAs: Put, Spd, and Spm. Results were expressed as nanomoles per gram of fresh weight (mean ± SE). Extractions from five different samples per treatment were made independently and each extract was quantified in duplicate.

Shoot Multiplication and Elongation in Partially Immersed System

For PIS, Growtek™ bioreactor (100×150 mm (Φ×h); Tarsons, India) with unique features like floating, rotating, nonabsorbing ex-plant holder with perforated ex-plant support matrix; side-tube with silicon rubber septum for changing media and online monitoring of medium environment was used. Each Growtek™ bioreactor was filled with 200 ml of modified MS medium (M3), MS medium supplemented with 1,238 mg l^{-1} NH_4NO_3, and 25 g l^{-1} sucrose (and for SM M3 gelled with 0.8% Agar) with varying concentration of naphthalene acetic acid (NAA; 5, 10, and 15 μM) and gibberellic acid (GA; 15, 30, 45, and 60 μM). Clusters of shoot buds (shoot bud length 0.5–1 cm) obtained from embryogenesis experiment were grown for a short period of 2 weeks on M2 medium and used as initial inoculum. Approximately 25 g of explant transferred in to both Growtek™ bioreactor and SM. The Growtek™ culture vessels were maintained at 25±2 °C under 16 h photoperiod, having illumination of 320 μmol $m^{-2}s^{-1}$ PFD on a gyratory shaker set at 80 rpm throughout the culturing period. Each treatment had at least 15 replications.

Rooting, Hardening, and Green House Cultivation

Rooting of the elongated Growtek™ bioreactor and SM grown banana shoots was achieved on MS medium with 1/2-strength NH_4NO_3 supplemented with NAA (1 mg l^{-1}) and 15 g l^{-1} sucrose (M4). Well-rooted plantlets were, then, planted in soil containing equiproportion of red soil, sand, and vermi-compost, which was found to be most suitable through our earlier trials.

Scanning Electron Microscopy

The samples treated with different PAs, consisting of primary or secondary explants, derived structures, and embryos at different developmental stages, were processed for scanning electron microscopy, according to Larry et al. [22]. The samples were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) for 6 h, dried in alcohol series up to 100%, sputter coated with gold, and examined in a LEO Scanning Electron Microscope 435 VP (Leo Electron Microscopy Ltd., Cambridge, UK).

Statistical Analysis

All data presented are the mean values of five replicates, and the data have been presented as means with standard errors of the means. All the observations and calculations were made separately. The data were analyzed by one-way analysis of variance, and post-hoc mean separations were performed by Duncan's Multiple Range Test at $P \leq .05$ [23].

Results and Discussion

Table 1 summarizes the endogenous contents of PAs in cultures grown on M2 medium where all the PAs increased with time. Such time-dependent accumulation of PAs and higher accumulation of PAs by organized cultures have also been observed in oat [24], in red spruce [25] and embryogenic suspension cultures of *Araucaria angustifolia* [15]. The higher accumulation of Spm is probably due to the stress condition towards the end of culture period where nutritional stress could play a significant role. Stress-dependent increase in the levels of Spm has been observed in various other studies [17, 15]. The cultures on M2 medium remained mostly unchanged except for a slight change in shoot height. When the external PAs were fed to these cultures, there were significant changes in the response, both in terms of culture morphology as well as levels of endogenous levels of PAs (Fig. 1). There was no significant difference of treatment with low levels (10 μM) of any PA from that of control. Both Spd and Put at 30 μM showed significant changes in morphology where Spd induced fluffy callus interspersed with embryonic masses, and browning of medium was observed whereas Put, at similar levels, resulted in higher nonembryogenic coherent callus with lesser embryogenic masses, although medium browning was not observed. Spm, though at 30 μM, did not show any significant difference from that of control; a higher level (40 μM) showed very noteworthy effect resulting in enhancement of highly embryogenic culture mass. Further higher levels such as 50 μM of all the PAs showed browning of medium leading to the culture necrosis followed by death.

A detailed investigation of morphological events occurring towards embryogenesis in Spm (40 μM)-treated cultures was recorded using scanning electron microscope. There were sequential events occurring towards embryogenesis, starting from globular masses passing though further stages of pro-embryos and embryos at various stages of development towards shoot formation. These observations suggest the usefulness of PAs for enhancing embryogenic events in in vitro cultures of banana. Contrarily, in other studies, Put was more useful in supporting organized cultures of oat (*Avena* sp.) and its cultures obtained after reciprocal crosses [24].

There were much higher differences in the pools of endogenous PAs as a consequence of feeding external PAs (Table 1). Cultures treated with Spm (40 μM) showed a high endogenous level of Spm accounting for 2,500 nmol gFW^{-1} upon 12 weeks of culturing due to gradual accumulation throughout the culture period (Table 1). The next significant effect was that of 30 μM of Put, which invariably increased only endogenous Put pools, which was also highest (1,000 nM) on 12 weeks, although periodic fluctuations were observed during the culture period (Table 1). Spd, supplied at 30 μM, though increased Spm pools (400 nmol g FW^{-1} on 8 weeks of culturing), contributed for the increase in levels of other PAs as well (Table 1). Variable results have been observed in various other studies upon external applications of PAs. For example, the application of Put (20 μM) and Spd (40 μM) for callus cultures of *Pinus oocarpa* and *Pinus patula* stimulated the embryogenesis [26] as is the case of the present study. Contrarily, during the growth of

Table 1 Levels of endogenous total polyamines in banana cultures grown on Basic medium (M2) with different levels various polyamines.

Treatments	Spermidine (nmol gFW^{-1})			Putrescine (nmol gFW^{-1})			Spermine (nmol gFW^{-1})		
	Weeks after inoculation								
	4	8	12	4	8	12	4	8	12
Control Medium (M2)	4.14 d	7.04 d	13.44 d	15.46 cd	17.44 cd	112.17 cd	28.39 c	86.10 cd	393.88 c
M2 + 40 μM Spermidine	95.02 bc	129.00 bc	92.85 bc	224.75 bc	339.08 ab	62.51 cd	396.98 bc	412.30 bc	202.88 c
M2 + 30 μM Putrescine	48.92 c	125.82 bc	45.06 c	629.00 a	459.67 a	991.37 a	75.80 c	97.05 cd	56.31 cd
M2 + 40 μM Spermine	247.39 a	368.14 a	237.40 a	442.51 b	441.31 a	564.89 b	1954.77 a	2120.52 a	2542.82 a

Data presented as mean of five replicates. Means with common letters are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test

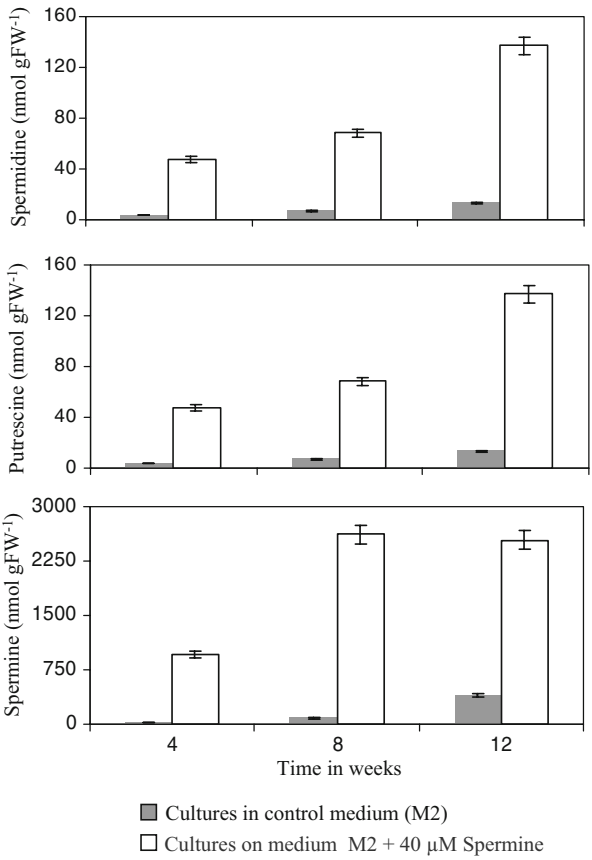

Fig. 1 Comparison of total endogenous polyamines culture segments grown in control medium (M2; *filled bar*) and those grown in M2 + 40 μM Spermine (*unfilled bar*). Data presented as mean of five replicates ± SE

embryogenic suspension cultures of *Pinus taeda*, high levels of endogenous Put were associated with reduction in cell division whereas in oats, Put enhanced the formation of somatic embryos [24]. In *Pinus sylvestris*, Spd retarded cell proliferation and growth but enhanced somatic embryo maturation [26]. Thus, in most of the cultures, these free exogenous PAs supported organization rather than unorganized growth. Accordingly, the PAs were higher in organized cultures, indicating that PAs are invariably organization-dependent.

In earlier studies, DFMO and DFMA have been noted to inhibit the endogenous pool sizes of PAs probably by inhibiting either the biosynthetic pathway or by degrading the accumulated pools of PAs to various degrees in different plant species. Therefore, in the present study, it was interesting to check to what extent these inhibitors reduce the pools of PAs in culture segments of banana having numerous regenerated shoot buds. These explants, being organized, showed almost similar levels of PAs as observed in embryogenic cultures mentioned earlier. When both DFMO and DFMA were used at 0.1 mM levels, most of the explants turned brown in 8 weeks and by the 12th week, shoot necrosis was apparent in all the treatments. When similar treatments were applied to embryogenic cultures, the already-formed embryos also necrosed and failed to form complete shoots. In these treatments, the endogenous pools of PAs were considerably disturbed with a higher suppression of all the PAs by DFMA (Fig. 2A) than by DFMO (Fig. 2B). The rate of

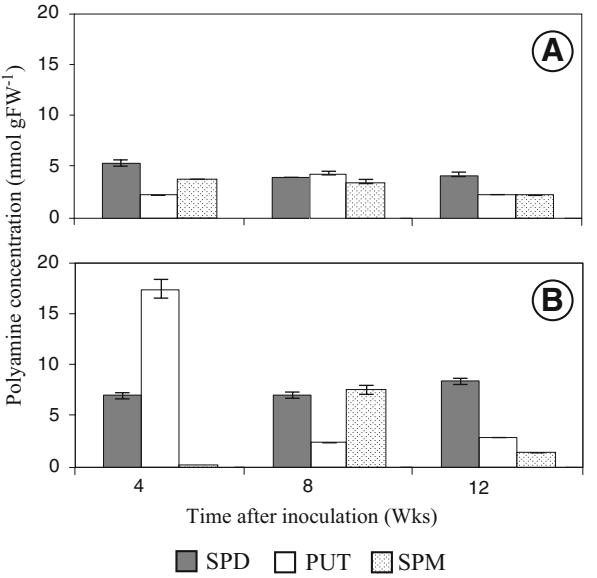

Fig. 2 Changes in total endogenous polyamines in cultures treated with polyamine inhibitors. **A** DFMA at 0.1 mM supplemented to basic medium (M2) containing 40 μM Spermine. **B** DFMO at 0.1 mM supplemented to basic medium (M2) containing 40 μM Spermine

suppression of Spd was negligible in both the inhibitor treatments whereas Put was periodically retarded by DMFA. These results confirm that the above effects are, in fact, due to the direct participation of externally fed PAs by way of cellular incorporation and not due to any other effects of indirect nature.

The accumulation of Spm in embryogenesis may be essential for the shift from callus to embryogenesis. A fine balance of different PAs may be required for embryogenesis. This is

Table 2 Effects of different levels of NAA and GA (supplemented through M3 medium) on height and number of shoots in two culture systems such as Solid Medium and Partial Immersion System grown for 2 weeks and 4 weeks.

Growth regulator (μM)	Shoot multiplication				Shoot elongation (cm)			
	SM		TIS		SM		TIS	
	Weeks after inoculation							
	2	4	2	4	2	4	2	4
NAA								
5	10 b	14 ab	8 b	10 c	2 d	3 d	3 e	7 e
10	12 a	16 a	6 c	12 b	3 e	4 c	5 cd	10 b
15	10 b	14 ab	10 a	14 a	2 d	3 d	4 de	8 cd
GA								
15	4 d	6 c	3 d	4 f	3 c	4 c	6 bc	10 b
30	7 c	12 b	6 c	8 d	4 b	7 a	10 a	12 a
45	6 c	8 c	5 c	6 e	5 a	6 b	7 b	9 bc
60	4 d	6 c	5 c	4 f	4 b	4 c	7 b	8 cd

Data presented as mean of five replicates. Means with common letters are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test

SM Solid medium, *PIS* partial immersion system

evident from our experimental results that treatment with inhibitors of PAs biosynthesis resulted in drastic reduction towards embryogenesis. Similar kind of response was observed in different plant systems with regard to morphogenic responses [14]. PAs are reported to promote shoot multiplication and in vitro flowering in *Cichorium intybus* [27]. In the present study, exogenous supply of Spm resulted in the enhancement of embryogenesis, suggesting the promotive role of PAs for embryogenesis in banana. In an earlier study, Beatriz et al. [28] studied the effect of exogenous administration of PAs in the concentration range of 0, 50, and 100 μM. The results showed the occurrence of significant differences among embryos indicating that the effects of PAs are not only species dependent but also organ dependent.

NAA and GA have been very useful for rapid elongation of cultured shoots leading to the generation of high quality shoots fit for acclimatization in NR variety of banana [29]. Therefore, the effects of these growth regulators, in combination with two different culture conditions SM and PIS, were studied. Although only increase in growth and shoot height were expected, there was also multiplication as a result of carry-over effects of earlier medium; this parameter was also recorded in addition to shoot height. In SM, all the concentrations of NAA resulted in good shoot multiplication as well as increase in shoot height (Table 2). Ten micromolar was found producing better results than other levels in terms of higher number or height of shoots, especially when grown for 4 weeks. Whereas in PIS, higher number of shoots as well as shoot height were observed when compared with SM, although 10 μM of NAA was more effective for shoot multiplication (Table 2). GA was more efficient in supporting shoot multiplication in SM than in PIS, more so when the concentration was 30 μM, although this level did not support as much multiplication as in NAA treatment. Nevertheless, GA very significantly supported shoot height in PIS than in SM. Because shoot height is a decisive factor in better acclimatization of banana shoots [29], GA appears very useful in obtaining high quality shoots. The Growtek™ bioreactor, functioning on the principle of PIS, has been used for micropropagation of other plant species where it was observed to support constant supply of nutrients and aeration to plants combining the advantages of both solid and liquid medium. The results of the present study also showed better utilization of nutrients in PIS than in SM because the biomass produced will be directly proportional to the nutrient utilization. All in all, almost similar multiplication with better shoot height were observed in PIS than in SM in a period of 4 weeks, suggesting that the observation made through the present study would be of great use for producing better quality shoots of NR banana. Uniform rooting in MS medium with 1/2-strength NH_4NO_3 supplemented with NAA (10 μM) was also observed. Such shoots appeared more robust than those from SM resulting in 100% survival in green house condition (data not shown). It is also worth mentioning that the use of Growtek™ bioreactor works out to be cheaper than any other commonly used apparatus meant for mass cloning of plants [18] because liquid form of culture medium is invariably cheaper than the gelled form with an added advantage of ease in handling.

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