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Development of an efficient micropropagation technique for *Musa* cv. Udhayam (ABB)

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ABSTRACT

Experiments were conducted to devise an efficient method of micropropagation for a high yielding but recalcitrant banana cv. Udhayam (Pisang Awak, ABB) using shoot tip explants. Virus-indexed shoot tips were established in medium comprising full-strength Murashige and Skoog (MS) basal salts and vitamins, supplemented with 50 mg l^{-1} ascorbic acid, 100 mg l^{-1} myo-inositol and 4.0 mg l^{-1} benzylamino purine (BAP). Among the various media tested for shoot proliferation, MS medium with BAP (3.0 mg l^{-1}) and 5% coconut water (CW) was found optimum as it produced the maximum number of 6.3 multiple shoots in a minimum period of 7.5 days. Rooting was achieved in the MS medium fortified with indole butyric acid (IBA) 0.5 mg l^{-1} + naphthalene acetic acid (NAA) 1.0 mg l^{-1} + activated charcoal (AC) 250 mg l^{-1}. Fully hardened planting material ensured high survival upon field transplantation. The development of a complete tissue culture protocol also includes the optimization of media and environment for successful primary and secondary hardening which has also been accomplished in the study.

Key words: Banana, recalcitrantce, Pisang Awak, micropropagation, shoot tip, direct organogenesis.

INTRODUCTION

The rapid spread of pests and diseases and need for clean and quality planting material has stimulated it's production through aseptic micropropagation techniques, *i.e.*, shoot tip culture (Macharia *et al*., 8). Besides the inherent advantages of tissue cultured bananas, it also plays a vital role in exchange of germplasm, conservation, and rapid propagation of newly selected varieties and hybrids thereby hastening the process of selection by plant breeders. The major disadvantages of *in vitro* micropropagation is that it requires variety specific protocols for it to be a successful commercial venture and occurence of genetically aberrant plants due to somaclonal variation. *Musa* cv. Udhayam is a new high yielding selection of banana giving 40% higher yield than local Karpuravalli from National Research Centre for Banana (NRCB), Trichy, Tamil Nadu. This is a dessert variety, belonging to Pisang Awak subgroup with ABB genome. The major problems associated with this variety are high phenolic exudation, recalcitrancy for *in vitro* regeneration and very low shoot proliferation rate (< 2.0). This necessitated the development of a reliable complete plant micropropagation protocol specific for the cultivar. This involved four steps, *viz.*, primary establishment of shoot tip cultures, multiplication of regenerated shoots, rooting of shoots and plantlet hardening.

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MATERIALS AND METHODS

Experiments were conducted at Tissue Culture Laboratory of NRCB, Trichy to develop an improved genotype specific protocol for the *in vitro* multiplication of *Musa* cv. Udhayam (Pisang Awak, ABB). About 2-3 month-old, virus-free sword suckers weighing 0.75 to 1.5 kg with 90-100 cm height were collected from NRCB Farm, Podavur, Trichy. They were cleaned and brought to the laboratory where they were washed with Tween 20® (2-3 drops/litre of water) for 30 min. followed by rinsing in running water and then treated with fungicide 0.1% carbendazim for 1 h. The explants were soaked in ascorbic acid (0.1%) for 30 min followed by repeated rinsing (3-4 times) with distilled water. Healthy meristematic shoot tips measuring 5 cm^3 were taken to laminar air-flow cabinet and surface sterilized with 4% (v/v) sodium hypochlorite for 10 min. followed by $HgCl_2$ (0.1%) for 10 min. with sterile water rinsings in-between. The explant was reduced to a size of 1.5-2.0 cm^3 prior to culture in the establishment medium (M_1). The details of the various media used in the study are given in Table 1.

Each shoot was decapitated during first subculture, apical meristem was damaged by giving cross cuts and sub-cultured at regular intervals of three weeks on M_2 medium for 2-3 times. The shoot buds obtained after third or fourth subculture were transferred to shoot proliferation medium (M_3).

Table 1. Media used at various micropropagation stages in banana.

Code	Medium composition	Purpose
M_1	Full-strength MS basal salts (Murashige and Skoog, 1962) and vitamins, 20 mg l^{-1} ascorbic acid, 100 mg l^{-1} myo-inositol and 3.0 mg l^{-1} benzylamino purine (BAP) + sucrose 3% (w/v) + Gelrite® (0.2%)	Initial establishment
M_2	Full-strength MS basal salts and vitamins, 20 mg l^{-1} ascorbic acid, 100 mg l^{-1} myo-inositol and 4.0 mg l^{-1} BAP + sucrose 3% (w/v) + agar (0.7%)	Shoot bud initiation
M_3	Full-strength MS basal salts and vitamins, 50 mg l^{-1} ascorbic acid, 100 mg l^{-1} myo-inositol and 3.0 mg l^{-1} BAP and coconut water (CW) + sucrose 3% (w/v) + agar (0.7%).	Shoot proliferation
M_4	Half-strength MS basal salts and vitamins fortified with 50 mg l^{-1} ascorbic acid, 100 mg l^{-1} myo-inositol, 250 mg l^{-1} AC and growth regulators such as IBA and NAA in different combinations + sucrose 3% (w/v) + agar (0.7%).	Rooting

Enrichment of the culture medium with coconut water, which contains zeatin, a natural cytokinin is found to be beneficial at sub-optimal levels of cytokinin. Therefore, in the present study MS medium with BAP 3.0 mg l^{-1} and CW at different levels were tested. The coconut water was obtained by heating the tender coconut water at 80-100ºC for 10 min. with continuous stirring to precipitate out the proteins, fats and other materials. The precipitate was filtered and the filtrate is stored at -20ºC for further use. Coconut water is said to be complex in nature because of its indefinite composition which varies from nut to nut and often they do not produce reproducible results. Hence, this particular trial was repeated twice to ascertain their reproducibility. After a total of seven subsequent sub-cultures on M_3 medium, the individual shoots of 2-3 cm size were transferred onto rooting (M_4) medium. The pH of the medium was adjusted to 5.8 before autoclaving at 121ºC for 20 min. under 1.1 kg cm^{-2} pressure. The cultures were incubated at 26 ± 2ºC under 16/8 h photoperiod (2000-4000 lux) provided by cool white fluorescent tubes and 70% relative humidity (RH). After 40 days of culture in the rooting media, the plantlets with shoots of 8-10 cm length were removed carefully from the culture bottles and washed in running tap water. The plantlets were kept in portrays containing a wide variety of substrates namely cocopeat and cocopeat in combination with vermiculite, Soilrite® and sand to identify a suitable substrate for primary hardening. The primary hardening was carried out in two different conditions namely 50% shade net and plant growth chamber maintained at 27°C and 70% RH.

The primary hardened plants obtained from different trials were transplanted to black polybags (15 cm × 10 cm with 150 gauge thickness) containing sand, red earth and FYM (1:1:1) with and without the application of commercial AMF, *Glomus mosseae* @ 5 g per polybag. Drainage holes were made at the bottom of the polybags. The experiment was carried out in a shade net where the temperature was reduced by 2-3ºC than the ambient (28 ± 2ºC) and RH was maintained at 75% by the use of micro-sprinklers. After a period of 45 days, the plants were shifted to partial shade and maintained until field planting.

The observations, namely, days taken for multiple shoot initiation, number of multiple shoots produced per explant and length of each multiple shoot were recorded in M_3 medium from third sub-culture onwards. At each sub-culture, the shoots produced/explant and the shoot lengths were measured. During rooting phase, days taken for root initiation, number of roots, longest root length and plant height etc. were recorded. Plant height, number of leaves, number of roots, longest root length, collar girth (above 5 cm from the base of the plant) were recorded during primary and secondary hardening.

The experiment was laid out in a Completely Randomized Design (CRD) with ten for shoot proliferation, rooting and five replications for hardening trials. The results were analysed in a CRD using analysis of variance (ANOVA) of AGRES software. Means were separated using the LSD multiple range test at 5%.

RESULTS AND DISCUSSION

After 7-10 days of initiation in M_1 medium, the shoot tips of cv. Udhayam turned from creamy-white to greenish in colour and produced mostly single shoots indicating that the use of BAP alone was sufficient for the initial establishment of shoot tips of various banana cultivars (Gitonga *et al*., 5). Damaging of apical meristem resulted in the formation of visible adventitious shoot buds. Addition of ascorbic acid to the establishment (M_1) and shoot bud initiation (M_2) media prevented the oxidation of phenolic compounds, which not only prevents the development of explants but also leads to their death (Bhat and Chandel, 4).

The visible shoot buds obtained during the third or fourth sub-culture were further cultured individually on M_3 medium for shoot proliferation purpose. Appearance of several stunted tiny adventitious buds on the surface of fleshy bulbous structures is an indication of shoot proliferation. Significant differences existed among the various CW treatments for all the parameters studied. Among the various levels of CW tested, 5% (T1) was found optimum as it gave rise to the maximum number of multiple shoots (6.3) in a minimum period (7.5 days) (Figs. 1 & 2) as against the control where only three shoots were produced in 15 days. This might be attributed to the synergistic effect of the synthetic and natural cytokinins used in the shoot proliferation medium (Abeyratne and Lathiff, 1). The length of the multiple shoot was also maximum in T1. The cytokinins normally suppress the shoot length (Venkatachalam *et al.*, 17) contrarily in the present study, it had increased the shoot length. As the concentration of CW in the medium increased from 5 to 15%, there was a delay in the days taken for multiple shoot initiation with a simultaneous decline in the No. of multiple shoots produced per explant and shoot length.

Effect of media composition on days taken for multiple shoot initiation and number of shoots produced per explant was highly significant but their effect on the length of multiple shoot was statistically non-significant. Though addition of CW to the medium had a positive influence on shoot proliferation (Fig. 2) as the concentration increased beyond 5%, there was a delay in the multiple shoot initiation. This might be because of the supra optimal level of the cytokinin in the medium (Srangsam and Kanchanapoom, 13), explant type and the genetic configuration of the cultivar (Rahman *et al.*, 9). The number of sub-cultures was restricted to seven in order to reduce the possible occurrence of off types arising through somaclonal variation (Lee, 7). The tissue culture protocol developed in the present study resulted in the production of 150 plantlets in a period of eight months which was ten times more than those produced in the conventional method of propagation.

The success of any micropropagation technique depends on the survival and establishment during hardening and it largely depends on the formation of effective roots during *in vitro* rooting of shoots. So attempts were made in the present study for better rooting of 'Udhayam' plantlets derived from shoot tip culture. After 3-4 subcultures on M_3 medium, the individual shoots were cultured in MS medium containing a strong (NAA) and a weak (IBA) auxin along with activated charcoal to test their efficacy on rooting of Udhayam banana. Activated charcoal @ 250 mg l^{-1} was included invariably in all the treatments because besides acting as an antioxidant it also promotes the root growth and development in banana (Gubbuk and Pekmezci, 6). Highly significant differences existed among the various treatments for all root parameters studied except for shoot parameters like plant height and No. of leaves (Table 2). In rooting medium, at a lower concentration of NAA, as the concentration of IBA was increased from 0.5 to 2.0 mg l^{-1}, there was a reduction in the days taken for root initiation but the response was *vice versa* in the medium containing higher concentrations of NAA. This might be due to the uptake and requirement of auxins, which varies with the culture conditions, genotype, physiological and developmental stages of the shoots (Roland and von Arnold, 11). Among the various treatments tested, T5 which consisted of MS medium with IBA 0.5 mg l^{-1} + NAA 1.0 mg l^{-1} + AC 250 mg l^{-1} was found optimum as it produced

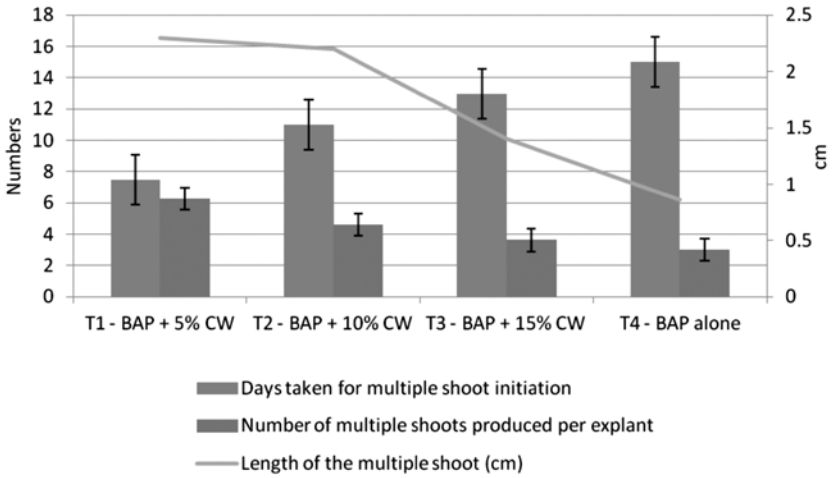

Fig. 1. Effect of different levels of coconut water on shoot proliferation in cv. Udhayam.

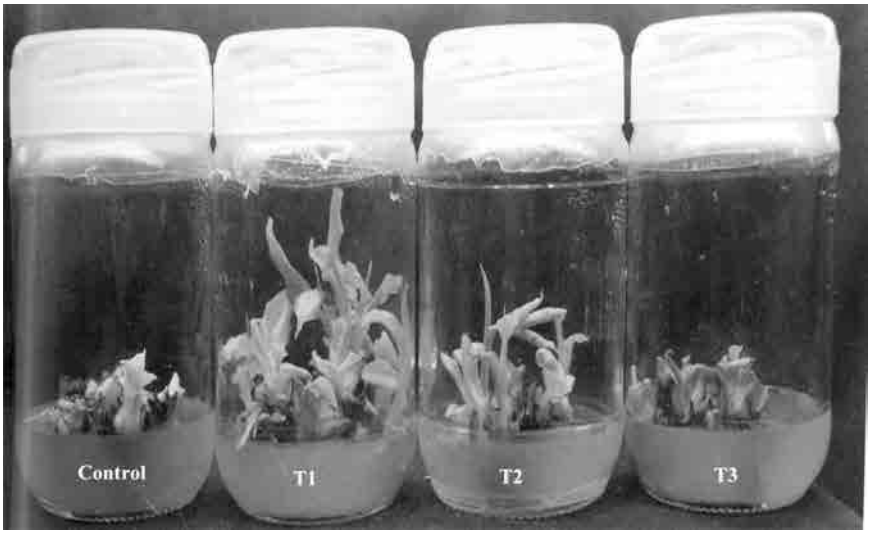

Fig. 2. Multiple shoot production from shoot tips cultured on MS medium supplemented with BAP 3.0 mg l^{-1} + different levels of coconut water. (Control = BAP alone; T1 = BAP + 5% CW; T2 = BAP + 10% CW; T3 = BAP + 15% CW)

Table 2. Effect different growth regulators and their combinations on rooting of shootlets in cv. Udhayam.

Treatment	Shoot height (cm)	Days taken for root initiation	No. of roots	Length of the longest root (cm)	No. of leaves
IBA (0.5 mg l^{-1}) + NAA (0.5 mg l^{-1})	0.93	15.50g	1.50bc	3.70b	1.62
IBA (1.0 mg l^{-1}) + NAA (0.5 mg l^{-1})	1.30	17.50h	1.62bc	5.20a	1.37
IBA (1.5 mg l^{-1}) + NAA (0.5 mg l^{-1})	1.49	14.50f	1.25bc	3.00d	1.40
IBA (2.0 mg l^{-1}) + NAA (0.5 mg l^{-1})	1.20	12.50d	1.75b	3.74b	1.25
IBA (0.5 mg l^{-1}) + NAA (1.0 mg l^{-1})	0.82	7.12a	4.75a	2.44e	1.50
IBA (1.0 mg l^{-1}) + NAA (1.0 mg l^{-1})	0.56	8.30b	1.2b5	3.35c	1.37
IBA (1.5 mg l^{-1}) + NAA (1.0 mg l^{-1})	0.98	13.50e	1.10c	1.80f	1.25
IBA (2.0 mg l^{-1}) + NAA (1.0 mg l^{-1})	0.22	10.75c	1.30bc	3.21cd	1.50
CD at 5%	10.67	0.60	0.51	0.26	0.51
Level of significance	NS	**	**	**	NS

Means within the same column followed by different letters are significantly different using Duncan's Multiple Range Test

the maximum number of roots (4.7) in a short period (7.7 days). In this experiment, IBA has proven its superiority over other auxins tested for root induction. This corroborates the results documented in several banana cultivars (Rahman *et al.*, 10). Further, the synergistic effect of NAA and IBA to produce functional roots have been demonstrated in the present study as reported in a variety of other crops like *Rosa* spp. (Singh and Syamal, 12), *Crateva magna* (Benniamin *et al.*, 3) etc. The positive influence of activated charcoal on rooting might be due to the adsorption of inhibitory substances in the culture medium, alteration of medium pH to an optimum level favouring growth and development and creation of a dark environment to simulate the soil conditions (Thomas, 16).

Good quality planting material with well developed roots and shoots could be easily hardened. Therefore, four different substrates and two different environments were tested for primary hardening of banana plantlet. Cocopeat is a lignin rich soil conditioner which needs less watering (up to 65%) and commonly used in hardening of tissue cultured bananas. Further, it prevents the hardening of potting soil and increases soil porosity. Hence, in the present study it was used alone and in combination with other substrates like vermiculite, soilrite® and sand which are likely to alter the physical composition of the media. Highly significant differences existed among the various treatments in both the environments for all root and shoot parameters studied except for No. of leaves

Table 3. Effect of different substrates and growing environments on primary hardening of *in vitro* raised plantlets of banana cv. Udhayam.

Treatment	Growth chamber				Net house			
	Plant height (cm)	No. of roots	Length of the longest root (cm)	No. of leaves	Plant height (cm)	No. of roots	Length of the longest root (cm)	No. of leaves
T1 [cocopeat + vermiculite]	2.73b	4.25a	8.70a	1.50	4.96a	8.00a	10.30a	1.50
T2 [cocopeat + soilrite]	2.95a	3.10b	6.50c	1.37	4.10b	3.10c	6.30b	1.25
T3 [cocopeat + sand]	2.64b	3.50b	6.90b	1.50	4.00b	4.00b	5.60c	1.37
T4 [control – cocopeat alone]	2.50c	2.62c	3.65d	1.37	2.00c	2.80c	4.20d	1.50
CD at 5%	0.08	0.48	0.09	0.53	0.21	0.72	0.52	0.52
Level of significance	**	**	**	NS	**	**	**	NS

Means within the same column followed by different letters are significantly different using Duncan's Multiple Range Test

produced (Table 3). Among the four substrates and two environments compared, the control (cocopeat alone) showed minimum growth, while cocopeat + vermiculite (1:1) showed the optimum growth and development in both the environments tested. The reason might be that cocopeat + vermiculite would have improved the water retention ability and aeration to the growing plant (Beardsell and Nicholas, 2) as well as altered anchorage and nutrient content of the medium thereby promoting the growth and development of nursery plants.

Among the two environments, shade net which is cost effective was superior over plant growth chamber in terms of plant height (5.0 cm), root number (8.7) and root length (10.5 cm). However, in shade net condition, the performance of Udhayam plantlets was better in cocopeat + vermiculite followed by cocopeat + soilrite® in terms of plant height and root length. But in growth chamber, T1 was best followed by T3 for all the growth parameters measured. However, they were statistically on par with each other.

The primary hardened plants were transferred to potting mixture containing equal proportion of sand, red earth and FYM enriched with and without commercial AM and maintained under 50% shade net for a period of 45 days. The per cent survival in the potting mixture without AMFwas 75% as reported by Abeyratne and Lathiff (1). Though AM increases the nutrient uptake, reduce disease incidence, improve water relations, chlorophyll content, drought tolerance and overall growth of micropropagated plants, their addition to the potting mixture did not have any significant influence on the growth and development of tissue cultured plants of Udhayam during secondary hardening. This is in agreement with the findings of Wang *et al.* (18) in *Gerbera* and *Nephrolepis*. However, their field establishment was high (95%) compared to control as reported by Strullu (14), which might be correlated with the greater spread of the mycelium in the soil benefitting the growth of each partner of the symbiotic team as reported by Thaker and Jasrai (15). This was quite evident in the present study as they showed an excellent survival, establishment and performance in the field.

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