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PROTOCOL FOR TISSUE CULTURE PROPAGATION OF BANANA CV. RAJAPURI BALE (AAB)

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ABSTRACT
Banana (*Musa* spp.) cv. Rajapuri Bale (AAB) is a popular cultivar of banana grown in Northern parts of Karnataka. Tissue culture propagation in banana is preferred for its faster multiplication rate compared to sucker propagation. The present study was carried out with the objective to investigate the effect of different ascorbic acid, BAP, IBA and NAA on *in vitro* regeneration. Shoot tip explants cultured on MS basal medium supplemented with ascorbic acid 175 mg/l, BAP 2.0 mg/l + NAA 0.25 mg/l and NAA 1.00 mg/l found effective in controlling browning, shoot multiplication and *in vitro* rooting, respectively in banana cv. Rajapuri Bale.

KEY WORDS: Rajapuri Bale, Tissue culture, Shoot tip, Ascorbic acid, Shoot multiplication.

INTRODUCTION

Banana and Plantains (*Musa* spp.) are one of the most valued fruit products. Banana belongs to the family *Musaceae* and section *Eumusa*. It signifies variable benefits, both as a staple food as well as a major export commodity for many tropical and subtropical countries. In general, banana cultivars are considered as good sources of carbohydrates, proteins, vitamins and minerals. Banana is known for its antiquity and it is interwoven with Indian heritage and culture. It is considered as the symbol of 'prosperity and fertility' owing to its greater socio-economic significance and multifaceted uses and high economic returns it is referred to as "Kalpatharu" (a plant of virtues) and Kalpavriksh[16]. Rajapuri Bale (AAB) is a popular cultivar of banana grown in Northern parts of Karnataka. It is a dwarf variety growing upto 6-8 feet height with a very thick stem and stands up very well to wind. The leaves are wider than those of most bananas growing upto 3 feet wide. It is the best plant to grow in marginal areas or where a grower does not intend to put much care into cultivation of bananas. The bunches weigh about 10-15 kg with 8-10 hands and 90-100 fingers. Fruits have attractive yellow colour with thick skin and good blend of sweet and acidity [13]. Bananas are generally propagated vegetative through suckers. Unfortunately, the traditional methods limited the expansion of bananas production due to a shortage of healthy plant material availability to farmers. High sterility of most cultivated bananas has historically prevented conventional breeding programs and plant propagation. Moreover, the longer time required by bananas to generate makes it even more difficult to breed them [15]. The major limitation with sucker propagation is the transmission of harmful insects, nematodes and viral diseases to field grown suckers. To overcome these issues and enable rapid multiplication of economically important commercial varieties, *in vitro* propagation is a preferred alternative method. Shoot tip culturing for bananas, provides second advantages that coincide with the farmers demands including, increased multiplication rate, physiological uniformity and the availability of disease-free materials all year round [9]. However, Rajapuri Bale is highly recalcitrant to tissue culture propagation mainly due to exudation of phenolic compounds from tissues and browning of the culture medium. This process is initiated by browning of the surface of plant tissues due to the oxidation of phenolic compounds resulting in the formation of quinines which are highly reactive and toxic to plant tissue. The browning phenomenon occurs in response to oxidation process of released phenolic compounds from injured tissue by phenol oxidase and formation of quinones [4]. Quinones negatively inhibit cell growth and can often result in death of cells (necrosis) [11]. Therefore, preconditioning of explants with media supplements such as ascorbic acid [7] was necessary to limit the production of these harmful substances. Keeping the above points in view, the present investigation "Protocol for Tissue Culture Propagation in Banana *cv*. Rajapuri Bale (*Musa* spp., AAB Group)" was carried out with the objective of minimization of browning, enhancement of shoot proliferation and induction of adventitious roots.

MATERIALS & METHODS

Preparation of explants

The present study was conducted at the Center for Horticulture Biotechnology, University of Horticultural Sciences, Bagalkot, India. Healthy and vigorously growing sword suckers of *cv*. Rajapuri (3-4 month age), free from viruses and other diseases were selected as a source of explant. Suckers were washed thoroughly under running tap water for 10-15 min. the suckers were then chopped off about 5-6 cm in length and 3-4 cm in diameter (fig. 1).

FIGURE 1. Mother plant and sword suckers of *cv*. Rajapuri Bale

The plant material obtained from the field was thoroughly washed in running tap water followed by washing with a detergent solution to remove adhering soil particles. Later, rhizomes were kept immersed in a fungicide solution of 1 % bavistin for half an hour, to further clean the planting material. The outer leaves, leaf base and corm tissue were trimmed using a sterilized stainless steel knife until the length of explant was 4-6 cm and the diameter, 3-4cm. These trimmed suckers enclosing the shoot tip were washed with double distilled water. After trimming one more outer layer, they were soaked in a solution of 0.50 % bavistin + 0.05% streptocycline for eight hours. After thoroughly washing with double distilled water, they were trimmed again, so that trimmed suckers were of 2-3 cm in length and 2-2.5 cm in diameter. These shoot tips were soaked in 0.05 % cetrimide for 30 minutes. After removing one more layer, the shoot tips were surface sterilized with 0.1% mercuric chloride in a closed container for 10 minutes. Further operations such as washing several times with sterile distilled water to remove all traces of chlorine, trimming of explants and inoculation were carried out under laminar air flow chamber (Fig. 2 A-C).

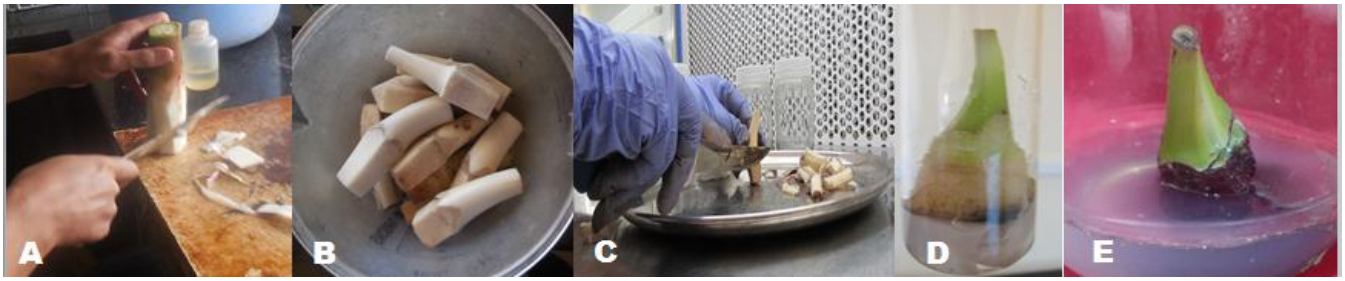

FIGURE 2. Establishment of aseptic culture: A) Trimming of suckers; B) Trimmed shoot tip; C) Trimming of shoot tip under laminar airflow; D) Inoculated shoot tips on liquid MS media; E) Established shoot tip.

Initiation of aseptic culture

Shoot tip explants were inoculated in MS liquid medium containing 2 mg/l BAP, 35 mg/L adenine sulphate and Different concentration of ascorbic acid concentration (25, 50, 75, 100, 125, 150,175, 200 mg/l) (Fig. 2D) for two weeks maintaining standard culture conditions of 25 $\pm 2^0$C temperature, 70 % RH and photoperiodic cycle of 16 hours light and 8 hours dark period. After two weeks of incubation, all the explants were evaluated for their percent response, browning and percent contamination in liquid medium. Greening and swelling of the explants were utilized as important criteria for assessing the success in establishment (Fig. 2E). Shoot tips that had turned dark brown/black and which did not swell were considered as non-established. Healthy and contamination free explants were excised by removing discolored tissue and transferred to the semisolid medium supplemented with BAP (2 mg/l) and adenine sulphate (35 mg/l) and incubated for four weeks maintaining standard culture conditions. The browning degree in initiation stage was scored visually as: +++ = High browning; ++ = Moderate browning and - = No browning.

Effect of cytokinin andauxin on shoot proliferation in Rajapuri Bale (AAB)

Established aseptic cultures were transferred onto multiplication media for shoot multiplication and development. The medium used for banana multiplication was Murashige & Skoog Medium (MS)[6]. Different concentration of 6-Benzyl amino urine (BAP), Naphthalene acetic acid (NAA) hormones were used for shoots induction and shoots multiplication. Individual shoots were inoculated onto half strength MS medium containing different concentration of Indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA) and activated charcoal 2 mg/l. All the media were autoclaved at 15 psi and 121 ˚C for 20 minutes.

The culture jars containing explants were incubated in growth chamber at 26 ±2°C with 16 hour photoperiod (approximately 2000 lux) provided by cool white fluorescent tubes. The materials were 6 times sub cultured at a regular interval of four weeks onto same medium to produce multiple shoots. Observations were recorded of percent response, number of shoots per explants, length of shoot (cm) and number of leaves per shoot. The regenerated plantlets after developing sufficient root system were deemed ready to transfer in soil. The plantlets were carefully removed from the culture vessels. The roots of the plantlets were gently washed under running tap water to remove agar attached to the roots. Observations were recorded onper cent rooting, number of roots, length of primary roots and length of secondary roots. Immediately after washing they were transferred to protrays containing a sterilized cocopeat and kept under poly tunnel for weeks. Later plantlets transferred topoly bags containing sand, red soil and compost in 1:1:1 ratio (v/v). They were kept under shade house and sprayed with water regularly to maintain high humidity around the plantlets.

Statistical analysis

The data were taken at four week intervals after incubation. There were five explants for each treatment and three replications. Data recorded for different parameters were subjected to completely randomized design (CRD). Statistical analysis was done by (ANOVA) using software Wasp developed by ICAR Research Complex, Goa.

RESULTS & DISCUSSION

The effect of ascorbic acid on browning of medium

Browning phenomena is one of the most common problems associated with *in vitro* establishments of shoot tip explant. In the present investigation, the effect of antioxidant ascorbic acid on browning and growth of banana cv. Rajapuri Bale were studied.

TABLE 1: Effect of ascorbic acid on browning intensity in banana cv. Rajapuri Bale (AAB)

Treatments	Browning intensity
T_1: MS B + BAP 2 mg/l (Control)	+++
T_2: MS B + BAP 2 mg/l + Ascorbic acid 25 mg/l	+++
T_3: MS B + BAP 2 mg/l + Ascorbic acid 50 mg/l	+++
T_4: MS B + BAP 2 mg/l + Ascorbic acid 75 mg/l	+++
T_5: MS B + BAP 2 mg/l BAP+ Ascorbic acid 100 mg/l	+++
T_6: MS B + BAP 2 mg/l BAP+ Ascorbic acid 125 mg/l	++
T_7: MS B + BAP 2 mg/l BAP+ Ascorbic acid 150 mg/l	++
T_8: MS B + BAP 2 mg/l BAP+ Ascorbic acid 175 mg/l	-
T_9: MS B + BAP 2mg/l BAP + Ascorbic acid 200 mg/l	-

MS B: Murashige & Skoog Basal Medium

+++ = High browning; ++ = Moderate browning; - = No browning

High browning war observed with Ms medium without the ascorbic acid (Control) (Table 1 & Fig. 3 A). The browning intensity reduced with increase in the concentrations of ascorbic acid. There was no browning when the MS medium was supplemented with ascorbic acid 175 mg/l and 200 mg/l (Table 1& Fig. 3 B-C).

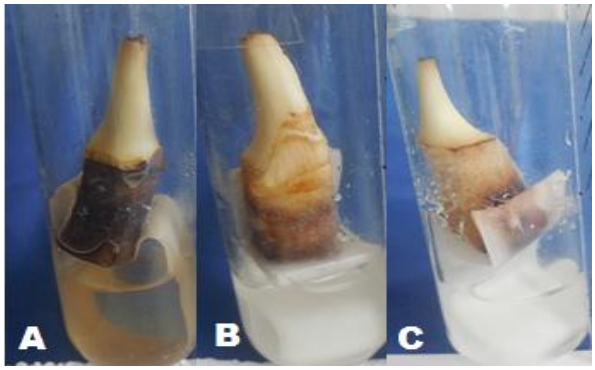

FIGURE 3. Intensity of browning in Rajapuri Bale on liquid MS medium: A) Control; B) Ascorbic acid 175 mg/l; C) Ascorbic acid 200 mg/l.

Highest browning intensity in control was attributed to oxidation phenols by oxygen radicals resulting in oxidative injury. Inhibition of browning at higher concentration of ascorbic acid was mainly due reducing activity of ascorbic acid, therefore, cells are protected from oxidative injury. Phenolic secretions and other exudates in plants tissue culture systems lessen the efficiency of explants initiation, growth and development [5]. One of the major problems for several tissue culture system, is the lethal browning which result in death of the cultured explants that depend on the rate of oxidation of phenolic compounds, as well as the quality of the total phenols [10].

Effect of cytokinin and auxin on shoot proliferation

TABLE 2: Effect of cytokinin and auxin on shoot growth in banana explants cv. RajapuriBale (AAB)

Treatments	Percent response	Number of shoots	Length of shoots (cm)	Number of leaves
T_1: MS B + BAP 2.0 mg/l (Control)	100 (89.53) *	3.61	4.33	3.61
T_2: MS B + BAP 2.0 mg/l + 2 weeks dark incubation + 1 Week light incubation	100 (89.53)	5.41	8.05	5.41
T_3: MS B + BAP 2.0 mg/l + NAA 0.25 mg/l	100 (89.53)	6.27	8.17	6.27
T_4: MS B + BAP 2.0 mg/l + NAA 0.50 mg/l	100 (89.53)	4.07	5.09	4.57
T_5: MS B + BAP 2.0 mg/l + NAA 0.75 mg/l	100 (89.53)	3.00	5.16	4.21
T_6: MS B + BAP 2.0 mg/l + NAA 1 mg/l	100 (89.53)	3.50	3.77	3.50
T_7: MS B + BAP 3.0 mg/l + NAA 0.25 mg/l	100 (89.53)	3.22	3.99	3.22
T_8: MS B + BAP 3.0 mg/l + NAA 0.50 mg/l	100 (89.53)	3.78	3.55	3.78
T_9: MS B + BAP 3.0 mg/l + NAA 0.75 mg/l	100 (89.53)	2.87	4.66	2.87
T_{10}: MS B + BAP 3.0 mg/l + NAA 1 mg/l	100 (89.53)	3.37	3.17	3.37
T_{11}: MS B + BAP 4.0 mg/l + NAA 0.25 mg/l	100 (89.53)	3.60	4.40	3.60
T_{12}: MS B + BAP 4.0 mg/l + NAA 0.50 mg/l	100 (89.53)	4.44	8.10	4.44
T_{13}:MS B + BAP 4.0 mg/l + NAA 0.75 mg/l	100 (89.53)	3.16	3.41	3.16
T_{14}: MS B + BAP 4.0 mg/l + NAA 1 mg/l	100 (89.53)	3.60	3.21	3.60
T_{15}: MS B + BAP 5.0 mg/l + NAA 0.25 mg/l	100 (89.53)	3.53	3.84	3.54
T_{16}: MS B + BAP 5.0 mg/l + NAA 0.50 mg/l	100 (89.53)	3.47	3.71	3.47
T_{17}: MS B + BAP 5.0 mg/l + NAA 0.75 mg/l	100 (89.53)	3.44	3.44	3.44
T_{18}: MS B + BAP 5.0 mg/l + NAA 1 mg/l	100 (89.53)	3.59	3.21	3.58
SE m ±	-	0.23	0.32	0.37
CD at 1%	NA	0.92	1.31	1.52

*Figures in parenthesis are arc sin transformation

Variable number of shoots were produced per explant in MS media supplemented with different concentrations of BAP and NAA. Among the different concentrations, MS B+ BAP 2.0 mg/l + NAA 0.25 mg/l showed significantly maximum number of shoot per explants (6.27) and the length of the shoots (8.17 cm) which was statistically on par with the treatment BAP 2.0 mg/l + 2 weeks dark incubation +1 Week light incubation (5.41 shoots per explant and 8.05 cm shoot length). Next best results were achieved with BAP 4.0 mg/l + NAA 0.50 mg/l which was superior as compared to control treatment (Table 2 & Fig. 4 A-D).

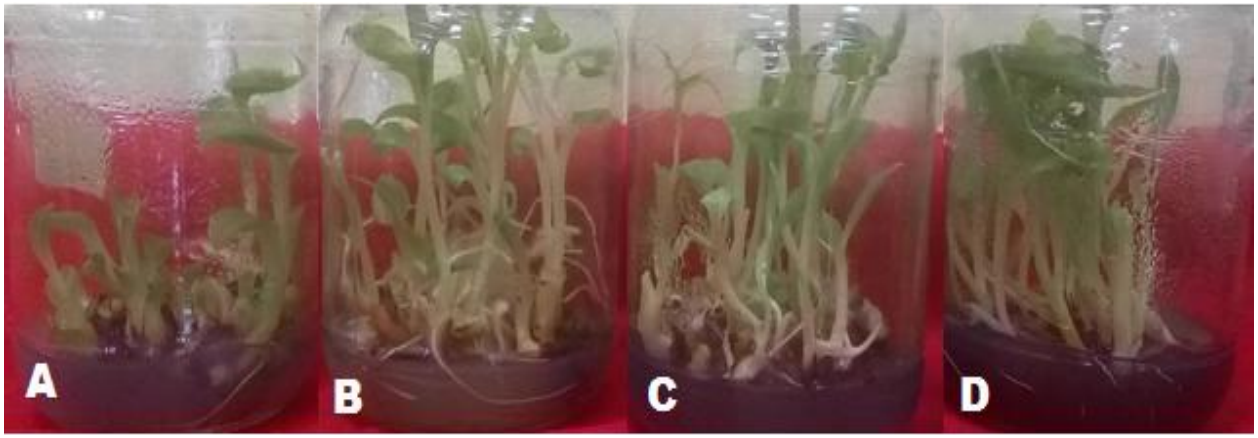

FIGURE 4. Shoot growth on media containing different concentration ofcytokinin and auxin: A) BAP 2.0 mg/l (Control); B) BAP 2.0 mg/l + NAA 0.25 mg/l C) BAP 2.0 mg/l + 2 weeks dark incubation + 1 Week lightincubation; D) BAP 4.0 mg/l + NAA 0.50 mg/l.

Enhanced shoot growth induced by BAP 2.00 mg/l + NAA 0.25 mg/l suggest that there was strong synergistic effect of BAP-NAA interaction. Similar findings were also reported by Srangsam and Kanchanapoom [17,8] in banana. The concentration and combination of auxin and cytokinin in the nutrient medium is key factor which determines successful plant regeneration [14]. Synergistic mechanism of cytokinin-auxin interaction in which cytokinin is required to activate a protein expressed in response to auxin [2].

Effect of auxins on rooting of microshoots cv. Rajapuri bale (AAB)

The data pertaining to the response of different auxins (IBA and NAA) on *in vitro* rooting of banana buds are presented in Table 3.

TABLE 3. Effect of auxins on *in vitro* rooting of banana plantlets cv. Rajapuri Bale (AAB)

Treatment (mg/l)	Percent response	Number of primary roots/plantlet	Length of primary roots (cm)	Number secondary roots/plantlet
T1: MS B + IBA 0.50	100 (89.41)*	2.07	5.63	9.10
T2: MS B + IBA 1.00	100 (89.41)	2.10	5.41	8.36
T3: MS B + IBA 1.50	100 (89.41)	2.67	7.73	8.55
T4: MS B + IBA 2.00	100 (89.41)	2.56	7.62	8.19
T5: MS B + NAA 0.50	100 (89.41)	2.14	5.45	6.44
T6: MS B + NAA 1.00	100 (89.41)	3.32	7.63	8.59
T7: MS B + NAA 1.50	100 (89.41)	1.82	4.33	6.83
T8: MS B + NAA 2.00	100 (89.41)	1.67	4.01	4.37
T9: MS Basal media (Control)	100 (89.41)	1.32	3.41	6.62
S.Em ±	-	0.12	0.19	1.23
CD@ 1%	NS	0.41	0.52	4.23

*Figures in parenthesis are arc sin transformation

Media containing different concentration of auxins showed variable effects on rooting. MS medium supplemented with NAA 1 mg/l showed maximum number of primary roots per shoots (3.32) and good length of primary roots (7.63 cm) and number of secondary roots (8.59), while, untreated control showed poor rooting (Table 3 & Fig. 5). Superiority of NAA may to be effective absorption, translocation and utilization as compared to other types of auxins [3]. These results are conformity with the findings of [1, 3 &12] in banana.

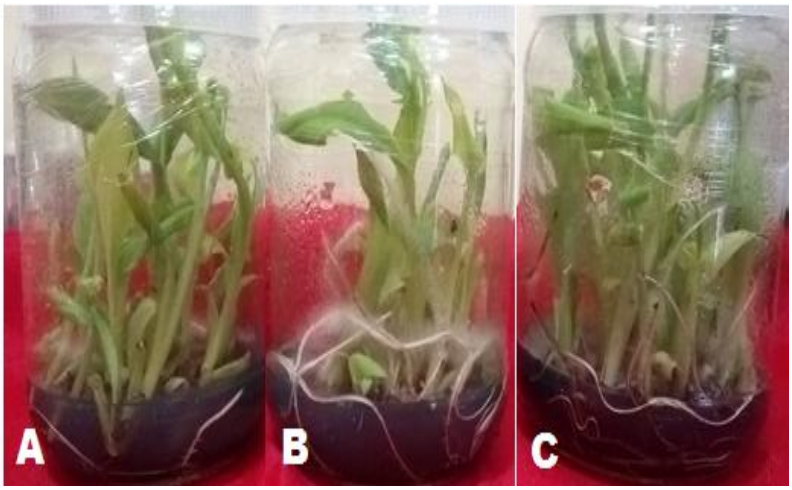

FIGURE 5. Effect of auxins on rooting: A) Control; B) MSB+ IBA 1.50 mg/l; C) MSB+ NAA 1.00 mg/l.

CONCLUSION

The findings of present study indicates that control of lethal phenolic browning, increased shoot proliferation and *in vitro* rooting could be achieved with MS medium +175mg/l ascorbic acid, MS medium + BAP 2.0 mg/l+ NAA 0.25 mg/l and half strength MS medium +NAA 1.00 mg/l. This protocol can be employed for large scale production of disease free planting material of banana cv. Rajapuri Bale.

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