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In Vitro Regeneration of Banana Cv. Nanjanagud Rasabale (AAB) by Shoot Tip Culture

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Authors' contributions

This work was carried out in collaboration with all authors. Author PG designed the study, managed the literature searches and wrote the first draft of the manuscript. Author RH performed the in vitro propagation study and statistical analysis. Author BAG managed the analysis of the study. All authors read and approved the final manuscript.

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ABSTRACT

The cultivar Nanjanagud Rasabale (*Musa* spp. AAB, Silk subgroup) geographical indication tagged and leading cultivar of Mysore district. It is known for unique taste, has a huge demand across the country. *In vitro* regeneration in banana is preferred for faster multiplication rate compared to sucker propagation. The present study was carried out with the objective to investigate the effect of different antioxidants, BAP and auxins on *in vitro* regeneration of banana cv. Nanjanagud Rasabale. Shoot tip explants cultured on MS liquid medium supplemented with ascorbic acid 50 mg/l was found effective in controlling browning. Enhanced shoot proliferation and shoot growth were observed with MS medium containing BAP 4.0 mg/l + NAA0.50 mg/l. *In vitro* rooting of microshoots was effective with half strength MS medium supplied with NAA 0.5 mg/l.

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Keywords: Nanjanagud Rasabale; in vitro; shoot tip; ascorbic acid; shoot multiplication.

ABBREVIATIONS

BAP : 6 Benzylaminopurine,
IBA : Indole-3-butyric acid,
NAA : Naphthalene acetic acid,
MS B : Murashige and Skoog's basal medium,
mg/l : Milligram per litre

1. INTRODUCTION

Banana and Plantain (*Musa* spp.) are widely grown in India with great socio-economic significance, interwoven in the cultural heritage of the country. Banana is fourth important food crop in terms of gross value exceeded by paddy, wheat and milk products. The cultivar Nanjanagud Rasabale (*Musa* spp. AAB, Silk subgroup) geographical indication tagged and once leading cultivar of Mysore district is under threat of extinction due to its susceptibility to panama disease [1]. The cultivar grown in around 600 acres of land in the district of Mysore, Karnataka, now confined to only 30 acres of land in isolated areas [2].

The Nanjangud Rasabale, known for its unique taste, has a huge demand across the country. But, growing conditions are not favorable enough for the cultivation of Nanjangud Rasabale and to match the huge demand that it generates [3]. This variety is susceptible to bacterial and viral diseases reducing the area of cultivation to only 5 ha, and hence it is considered as an endangered cultivar [4].

Bananas are generally propagated vegetatively through suckers. Unfortunately, the traditional methods limited the expansion of bananas production due to a shortage of healthy planting material availability to farmers. High sterility of most cultivated bananas has historically prevented conventional breeding programs and plant propagation [5]. 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* regeneration is a preferred alternative method. *In vitro* regeneration by shoot tip culture provides advantages of increased multiplication rate, physiological uniformity and the availability of disease-free materials all year round [6].

The main advantages of *in vitro* regeneration technique are rapid multiplication of plants with known desirable characters, free from pest and diseases, high survival rate during field establishment, vigorous growth, retention of healthy leaves, uniform growth, shortened harvesting period and higher yields. However, most of the commercial laboratories are dealing with improved varieties such as Grande Naine, Williams and other clones of Cavendish group [7]. This biased attitude towards the Nanjangud Rasabale is due to inherent problems associated with it such as higher degree of culture browning, poor multiplication rates etc., under *in vitro* conditions. Of these phenolic browning is one of the major constraints for *in vitro* regeneration of Nanajangud Rasabale. The principle phenols in include doparnine, catechin, chlorogenic acid, cinnamic acid, hydroxybenzoic acid, rescorciol, progallic acid, salicylic acid, ferulic acid, vanillin, coumarin and p-coumaric acid [8]. These phenolic compounds are responsible for browning reactions and high mortality rate in tissue culture [9]. Elimination or minimization of phenolic browning is, therefore, an essential prerequisite to successful *in vitro* regeneration.

Keeping the above points in view, the present investigation "*in vitro* regeneration of banana cv. Nanjangud Rasabale (*Musa* spp., AAB Group) by shoot tip culture" was carried out with the objective to overcome problem of browning, enhance of shoot proliferation and *in vitro* rooting.

2. MATERIALS AND METHODS

2.1 Preparation of Explants

The present study was conducted at the Center for Horticulture Biotechnology, University of Horticultural Sciences, Bagalkot 587 104, Karnataka, India. Uniform sword suckers of 3-4 month age were selected as a source of explants from one year old healthy and vigorously growing mother plant of banana cv. 'Nanjangud Rasabale' (Fig. 1) maintained at banana mother block, University of Horticultural Science, Bagalkot.

The plant materials obtained from the field were thoroughly washed in running tap water followed by soaking in a detergent solution containing Tween-20 2-3 drops/l (Himedia) for 30 min to remove adhering soil particles. Later, rhizomes were kept immersed in a fungicide solution of 1 % carzim 50 (Cheminova India Ltd, Bharuch) for half an hour, to further clean the planting

Fig. 1. Mother plant (1 year old) and sword suckers of banana cv. 'Nanjangud Rasabale '

material. The outer leaves, leaf base and corm tissue were trimmed using a sterilized stainless steel knife to get uniform size explants of 6-8 cm length and the diameter, 3-4 cm. 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% carzim 50 + 0.05% K-Cycline (Karnataka Antibiotics & Pharmaceuticals Limited, Bengaluru) for eight hours. After thorough washing with double distilled water, they were trimmed again, so that the explants were of 2-3 cm in length and 2-2.5 cm in diameter. These shoot tip explants were soaked in 0.05% cetrimide (Himedia) for 30 minutes. After removing one more layer, the shoot tip explants were surface sterilized with 0.1% mercuric chloride (Himedia) 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).

2.2 Initiation of Aseptic Culture

Shoot tip explants were inoculated onto MS liquid medium containing 2 mg/l BAP (Himedia), 35 mg/l adenine sulphate (Himedia) and different concentration of ascorbic acid (Himedia) (25, 50, 75, 100, 125, 150,175 mg/l; T_1- T_8) (Fig. 2 D) for two weeks. Cultures were maintained under standard culture conditions of 25 ± 3°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 percentage response, browning and percentage contamination in liquid medium. Greening and swelling of the explants were utilized as important criteria for assessing the success in establishment (Fig. 2 D). Shoot tips that had turned dark brown/black and which did not swell were considered as non-established. The browning degree in initiation stage was scored visually as: +++ = High browning; ++ = Moderate browning and - = No browning.

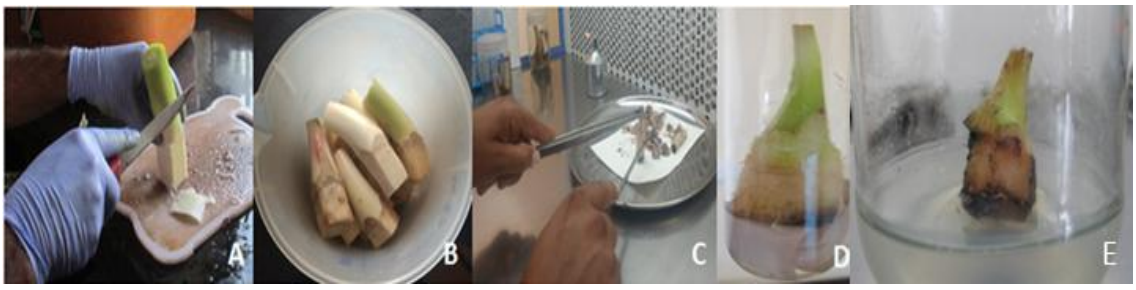

Fig. 2. Establishment of aseptic culture of 3-4 month age of explants : A) Trimming of suckers; B) Trimmed shoot tip explants after cetrimide treatment; C) Isolation of shoot tip explants under laminar airflow chamber; D) Established shoot tip culture onto MS liquid medium with filter paper support; E) Aseptic shoot tip culture established on MS semisolid medium

2.3 Effect of Cytokinin and Auxin on Shoot Proliferation

Established moderate and non-browned aseptic cultures (Fig. 2 E) were transferred onto multiplication media for shoot multiplication and development. The medium used for banana multiplication was Murashige & Skoog medium (MS) [10]. Different concentrations of 6-Benzyl aminopurine (BAP) (2, 3, 4 and 5 mg/l) in combination with Naphthalene acetic acid (NAA) (Himedia) (0.25, 0.50, 0.75 and 1 mg/l; T_3- T_{18}) were supplemented to multiplication media for shoots induction and proliferation along with treatments MS B + BAP 2.0 mg/l (T_1) and MS B + BAP 2.0 mg/l + 2 weeks dark incubation + 1 week light incubation (T_2). The cultures were maintained under standard culture conditions for four weeks. Sub-culturing was carried out at four weeks for eight passage cycles. Observations on percentage response, number of shoots/explants, length of shoots (cm) and number of leaves/shoot were recorded at each passages and average was calculated.

2.4 Effect of Auxin on *in vitro* Rooting

Individual shoots were inoculated onto half strength MS medium containing different concentration of Indole-3-butyric acid (IBA) (Himedia) (0.50, 1, 1.50 and 2 mg/l; T_1- T_4), Naphthalene acetic acid (NAA) (0.50, 1, 1.50 and 2 mg/l; T_5 - T_8) and activated charcoal 2 mg/l. The cultures were maintained under standard culture conditions for four weeks. The plantlets were carefully removed from the culture vessels and roots of the plantlets were gently washed under running tap water to remove agar attached to the roots. Observations on percentage response, number of primary roots/plantlet, length of primary roots (cm) and number secondary roots/plantlet were recorded.

2.5 Hardening of Plantlets

The plantlets were transferred to protrays containing sterilized cocopeat and kept under poly tunnel for 4 weeks (Fig. 3 A). Later plantlets were shifted to polybags containing sand, red soil and compost in 1:1:1 ratio (v/v) and maintained under 50 % shade for eight weeks (Fig. 3 B).

2.6 Statistical Analysis

Completely randomized block design (CRD) was used for the analysis of data. Each treatment was replicated three times with 15 explants. The data were subjected to ANOVA by using software Wasp developed by ICAR Research Complex, Goa (http://www.ccari.res.in/waspnew.html). Critical difference values were tabulated at one per cent probability where “F” test was significant.

3. RESULTS AND DISCUSSION

3.1 Browning of Medium

Browning phenomena is one of the most common problems associated with *in vitro* establishments of shoot tip explant. The browning intensity was reduced with increase in the concentrations of ascorbic acid (Table 1 & Fig. 4 A-D). There was no browning when the MS medium was supplemented with ascorbic acid at 50 - 175 mg/l (T_3- T_8). Whereas, it was higher and moderate with untreated control (T_1) and ascorbic acid at 50 mg/l (T_2), respectively. Highest browning intensity in untreated control may be attributed to oxidation phenols by oxygen radicals resulting in oxidative injury. Inhibition of browning at higher concentration (50 mg/l and above) of ascorbic acid may be attributed to its reducing activity thereby cells might have protected from oxidative injury. These findings are in conformity with the reports of [11] and [9]

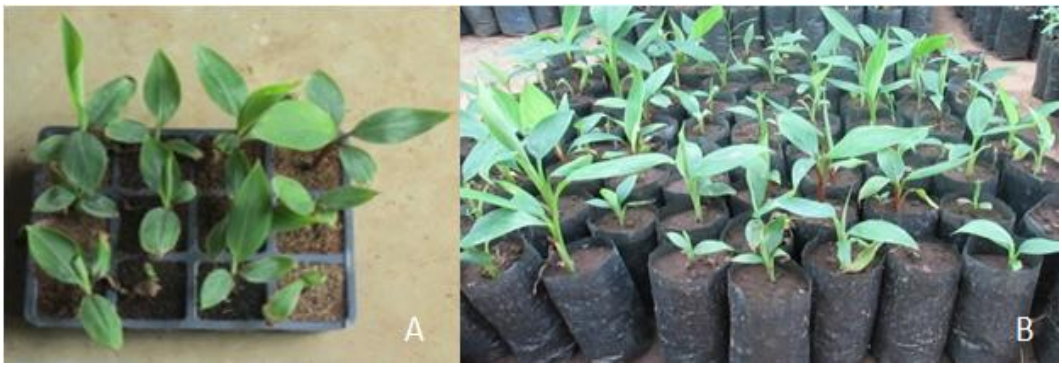

Fig. 3. A) Primary hardening (4 weeks); B) Secondary hardening (8 weeks)

in Banana cv. Barngan and Formosana, respectively. [12] opined that the phenolic secretions and other exudates in plants tissue culture systems lessen the efficiency of explants initiation, growth and development. 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 [13]. [14] reported that the ascorbic acid is able to scavenge oxygen radicals produced when plant is wounded, therefore, protecting the cells from the damage resulting from the injury. Further the detoxification of the free radicals by ascorbic acid produced through oxidation of the phenolic compounds reduces the extent of browning.

3.2 Shoot Proliferation and Growth

Variable number of shoots were produced per explant in MS media supplemented with different concentrations of BAP and NAA (Table 2 & Fig. 5 A-D). Among the different treatments, MS medium supplemented with BAP 4.0 mg/l + NAA 0.50 mg/l (T_{12}) showed significantly maximum number of shoot/explants (4.62), highest length of the shoots (6.10 cm) and good number of leaves per shoot (5.31) which was statistically on par with the treatment MS medium containing BAP 2.0 mg/l + NAA 1mg/l (T_6) (4.20 shoots/explants, 5.36 cm shoot length and 5.41 number of leaves per shoot). Next best results were achieved with the treatment MS medium + BAP 2.0 mg/l + 2 weeks dark incubation + 1 week light incubation (T_2) (3.41shoots/explants, 4.25 cm shoot length and 4.32 number of leaves/ shoot) and MS medium + BAP 2.0 mg/l (T_1) (3.40 shoots/explants, 4.04 cm shoot length and 4.01 number of leaves/shoot).

Enhanced shoot growth induced by the treatment, T_{12} suggest that there was strong synergistic effect of BAP-NAA interaction. Similar findings were also reported by [15,16] in banana. The concentration and combination of auxin and cytokinin in the nutrient medium is key factor which determines successful plant regeneration [17]. [18] opined that synergistic mechanism of cytokinin-auxin interaction in which cytokinin is required to activate a protein expressed in response to auxin.

Table 1. Effect of ascorbic acid on browning intensity

Treatments	Browning intensity
T_1: MS B + BAP 2 mg/l (Untreated 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 + Ascorbic acid 100 mg/l	-
T_6: MS B + BAP 2 mg/l + Ascorbic acid 125 mg/l	-
T_7: MS B + BAP 2 mg/l + Ascorbic acid 150 mg/l	-
T_8: MS B + BAP 2 mg/l + Ascorbic acid 175 mg/l	-

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

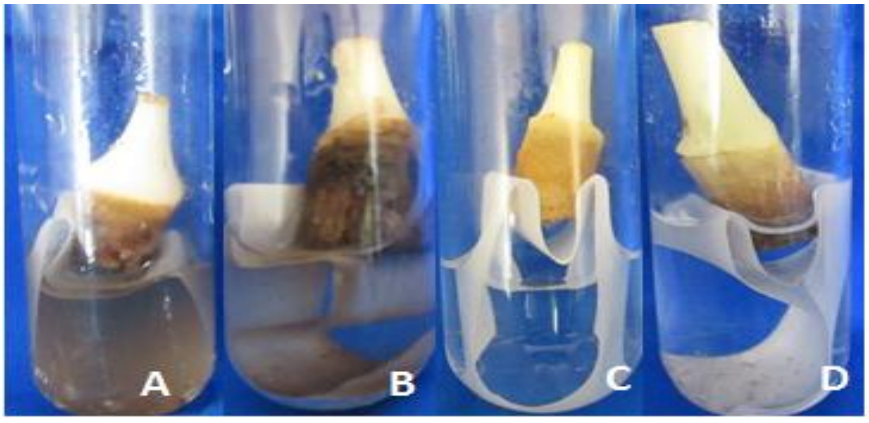

Fig. 4. Browning intensity (15 days old): A) Untreated control; B) Ascorbic acid 25 mg/l; C) Ascorbic acid 50 mg/l D) Ascorbic acid 75 mg/l

Table 2. Effect of cytokinin and auxin on shoot proliferation and growth

Treatments	Percentage response	Number of shoots/explants	Length of shoots (cm)	Number of leaves/shoot
T_1: MS B + BAP 2.0 mg/l	100 (89.53) *	3.40	4.04	4.01
T_2: MS B + BAP 2.0 mg/l + 2 weeks dark incubation + 1 week light incubation	100 (89.53)	3.41	4.25	4.32
T_3: MS B + BAP 2.0 mg/l + NAA 0.25 mg/l	100 (89.53)	2.27	3.21	3.22
T_4: MS B + BAP 2.0 mg/l + NAA 0.50 mg/l	100 (89.53)	3.07	3.19	3.57
T_5: MS B + BAP 2.0 mg/l + NAA 0.75 mg/l	100 (89.53)	3.22	3.24	3.21
T_6: MS B + BAP 2.0 mg/l + NAA 1 mg/l	100 (89.53)	4.20	5.36	5.41
T_7: MS B + BAP 3.0 mg/l + NAA 0.25 mg/l	100 (89.53)	3.11	3.31	3.53
T_8: MS B + BAP 3.0 mg/l + NAA 0.50 mg/l	100 (89.53)	3.23	3.55	3.68
T_9: MS B + BAP 3.0 mg/l + NAA 0.75 mg/l	100 (89.53)	2.52	3.52	2.99
T_{10}: MS B + BAP 3.0 mg/l + NAA 1 mg/l	100 (89.53)	3.36	3.61	3.49
T_{11}: MS B + BAP 4.0 mg/l + NAA 0.25 mg/l	100 (89.53)	3.39	3.64	3.77
T_{12}: MS B + BAP 4.0 mg/l + NAA 0.50 mg/l	100 (89.53)	4.62	6.10	5.31
T_{13}:MS B + BAP 4.0 mg/l + NAA 0.75 mg/l	100 (89.53)	3.03	3.53	3.44
T_{14}: MS B + BAP 4.0 mg/l + NAA 1 mg/l	100 (89.53)	3.41	3.55	3.26
T_{15}: MS B + BAP 5.0 mg/l + NAA 0.25 mg/l	100 (89.53)	3.49	3.84	3.51
T_{16}: MS B + BAP 5.0 mg/l + NAA 0.50 mg/l	100 (89.53)	3.58	3.81	3.31
T_{17}: MS B + BAP 5.0 mg/l + NAA 0.75 mg/l	100 (89.53)	3.51	3.36	3.63
T_{18}: MS B + BAP 5.0 mg/l + NAA 1 mg/l	100 (89.53)	3.62	3.53	3.52
S. E m ±	**-**	**0.30**	**0.41**	**0.49**
CD@1%	**NA**	**0.93**	**1.36**	**1.54**

The values given in parenthesis are arc sine transformed values **(Sin-1 √X/100)*

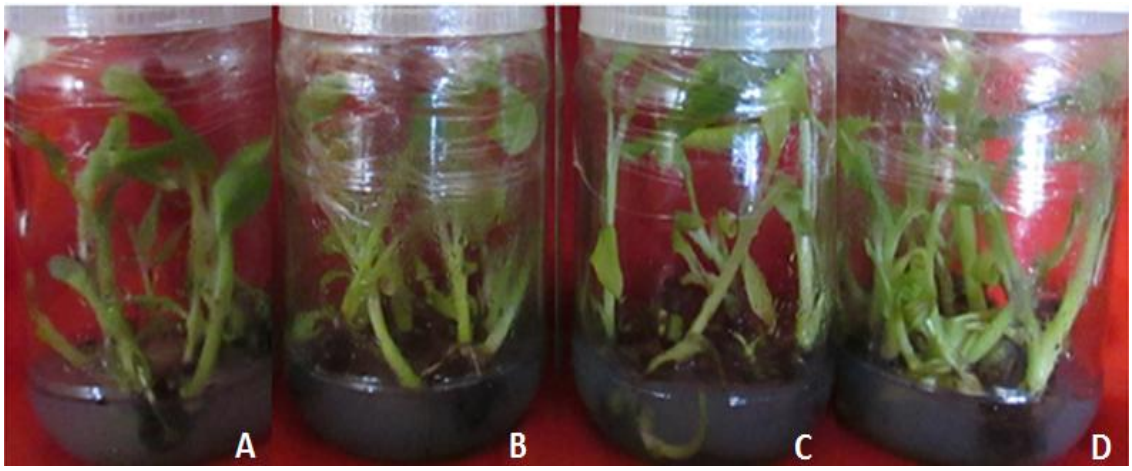

Fig. 5. Shoot proliferation and growth on MS media at 5th subculture cycle (5 month old culture): A) BAP 2.0 mg/l (Control); B) BAP 2.0 mg/l + 2 weeks dark incubation + 1 week light incubation; C) MS B + BAP 2.0 mg/l + NAA 1 mg/l; D) BAP 4.0 mg/l + NAA 0.50 mg/l

3.3 *In Vitro* Rooting of Microshoots

Media containing different concentration of auxins showed variable effects on *in vitro* rooting (Table 3 & Fig. 6). MS medium supplemented with NAA 0.50 mg/l induced significantly maximum number of primary roots/plantlet (3.42) and good length of primary roots (6.73 cm) and number of secondary roots/plantlet (8.51). While, untreated control showed poor rooting (1.54 primary roots/plantlet, 3.62 cm length of primary roots and 5.96 secondary roots/plantlet). It is evident that NAA was effective in inducing adventitious roots at lower concentrations. Superiority of NAA may be due to its effective absorption, translocation and utilization as compared to other types of auxins [19]. These results are in conformity with the findings of [20, 19,21] in banana. The rooting media were supplemented with activated charcoal which might have also influenced the morphogenesis by the irreversible absorption of inhibitory compounds in the culture medium leading to substantial decrease in the metabolites, phenolic exudation and accumulation of brown exudates. Similar observations were made by [22] in various horticulture crops during orchid seed germination, somatic embryogenesis, anther culture, synthetic seed production, protoplast culture, stem elongation, etc.

Table 3. Effect of Auxins on *in vitro* rooting

Treatment	Percentage response	Number of primary roots/plantlet	Length of primary roots (cm)	Number secondary roots/plantlet
T_1: MS B + IBA 0.50 mg/l	100 (89.53)*	2.13	5.21	7.10
T_2: MS B + IBA 1.00 mg/l	100 (89.53)	2.17	5.06	7.14
T_3: MS B + IBA 1.50 mg/l	100 (89.53)	2.72	7.82	8.64
T_4: MS B + IBA 2.00 mg/l	100 (89.53)	2.31	6.68	7.89
T_5: MS B + NAA 0.50 mg/l	100 (89.53)	3.42	6.73	8.51
T_6: MS B + NAA 1.00 mg/l	100 (89.53)	2.32	4.51	6.81
T_7: MS B + NAA 1.50 mg/l	100 (89.53)	1.83	4.19	6.43
T_8: MS B + NAA 2.00 mg/l	100 (89.53)	1.72	4.82	4.21
T_9: MS Basal media (Untreated control)	100 (89.53)	1.54	3.62	5.96
S.Em ±	-	0.04	0.13	0.39
CD@ 1%	NS	0.14	0.42	1.09

**The values given in parenthesis are arc sine transformed values (Sin-1 √X/100)*

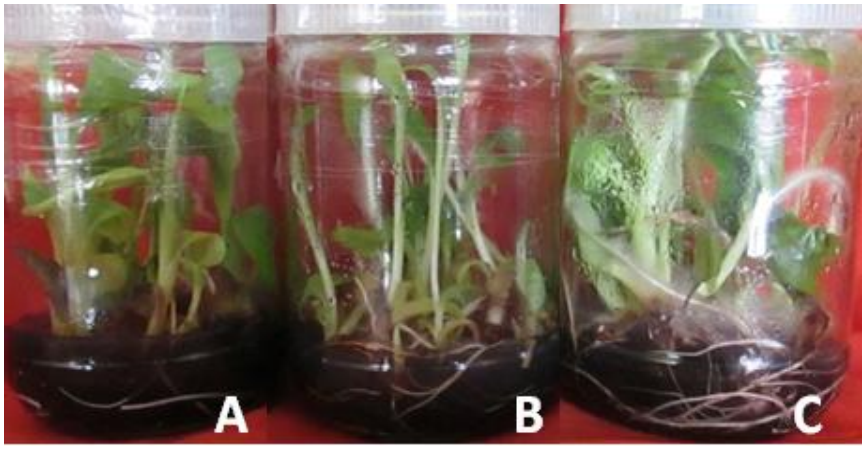

Fig. 6. *In vitro* rooting of microshoots on half strength MS media 4th week: A) Control; B) IBA 1.50 mg/l; C) NAA 0.50 mg/l

4. CONCLUSION

The findings of present study demonstrate that the control of lethal phenolic browning, increased shoot proliferation and *in vitro* rooting could be achieved with MS liquid medium supplemented with 50 mg/l ascorbic acid, MS medium containing BAP 4.0 mg/l+ NAA 0.50 mg/l and half strength MS medium + NAA 0.50 mg/l, respectively. This protocol could be employed for large scale *in vitro* regeneration of disease free planting material of banana cv. Nanjangud Rasabale.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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