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An Efficient Protocol for *in vitro* Regeneration of Banana var. *Nanjangudu rasabale* (*Musa* spp. AAB)

P. Babu*

College of Agriculture, Hanumanamatti, UAS, Dharwad, India

**Corresponding author*

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A B S T R A C T

Banana variety *Nanjanagudu rasabale* has been found growing in parts of Mysuru district, known for its unique aroma, flavour, taste and shelf life but unfortunately deemed as an endangered variety. Having given GI protection hope for bringing this variety back to field by producing disease free tissue culture plants demanded development of an efficient protocol for *in vitro* regeneration. In the present investigation, both citric acid and ascorbic acid found effective in inhibiting browning of shoot tip explant due to phenolic compounds. Each of the chemical sterilants was effective in reducing microbial contamination when they were used in sequence one after the other.MS media supplemented with BAP at 3.0 mg/l took least number of days for shoot regeneration and favoured better shoot production with maximum number of leaves per shoot and shoot length. Addition of 25mg/l adenine sulphate and 2-3 ml of Aonla juice proliferated maximum number of shoots during multiplication. MS media at half strength provided with activated charcoal and IBA at 2 mg/l was effective in producing better roots from *in vitro* grown shoots.

Introduction

Banana belonging to the family Musaceae and section Eumusa, and the cultivated edible types are mainly triploid in nature with basic chromosome number 11 (Salaria, 2004).Banana is the world's most widely known and distributed fruit, eaten raw, cooked or processed. In general, bananas are good source of carbohydrates, proteins, vitamins and minerals. It is treated as symbol of prosperity and fertility owing to its greater socio-economic significance and utility, it is referred to as kalpatharu and kalpavriksh (Singh, 2009).

Banana cultivar *Nanjanagudu rasabale* (*Musa* spp. AAB) classified under silk subgroup has been given Geographical Indication (GI) protectionin 2005 under the Goods (Registration and Protection) Act, 1999 by Government of India for its distinguished aroma, flavour and taste. *Nanjanagudu rasabale* has been grown in and around Mysuru and Chamarajanagar districts of Karnataka and drives huge demand throughout the country. This variety is very difficult to get and becoming rarer by the day. Very few shops sell this varietyin Mysuru at an exorbitant price, but it is worth buying it.

Unfortunately, this variety is highly susceptible for *Fussarium oxysporum* var. *cubense* and the area under cultivation has been drastically reduced over last three decades from 500 acre to just 5ha and is regarded as an endangered variety (Lakshmanan *et al*., 2007).

High sterile nature of most cultivated varieties has restricted conventional breeding and plant propagation. Banana is generally propagated vegetative through suckers. Only 5 to 10 suckers can be obtained from a plant per year in conventional method. But problem with the use of suckers is the transmission of insects, nematodes and viral diseases. The traditional method of propagation is laborious; time consuming and not very efficient as far as production of homogenous plants is concerned (Banaerjee and De Langhe, 1985). This variety in particular, having a genome AAB it is difficult to get regenerate *in vitro* because of higher exudation of phenolic compounds.

In vitro propagation technique using shoot tip cultures is a necessary approach in dealing with the problems encountered in conventional propagation and in tissue culture. This technique will ensure sustainable production of banana planting materials. Most of the organised cultures, especially the shoot tips maintain strict genotypic and phenotypic stability under tissue culture conditions (Bennici, 2004).

A large number of uniform disease free plants can be produced in this method from a single plant or even a small plant tissue (explants) showing good genetic potential (Martin *et al*., 2006). Taking all these factors in to account, this experiment was executed to develop a standardised protocol to produce disease free genuine quality planting material required to satisfy the growers requirement.

Materials and Methods

Explants preparation

This study was carried out at the laboratory of Plant tissue culture, Department of Biotechnology, College of Agriculture, Vijayapura. Healthy free from diseases particularly fusarium infection and vigorously growing around 3-4 months old sword suckers of variety *Nanjanagudu rasabale* were collected from the farmer field at Devarasanahalli, Nanjanagudu taluk, Mysuru district. Suckers were cured for two days under shade and were washed under running tap water for about 30 minutes. Roots and leaf sheaths were removed using a sharp knife and they were peeled off to an approximate size of 4 cm at the base and 5 cm long with a single shoot tip. Further, they were taken in 2 % bavistin solution for 1-2 hours along with few drops of tween 20 and washed them for 30 minutes under running tap water to remove bavistin and teepol. Later, they were keptin to a solution containing1% Indofil M-45 for 15-20 minutes. Further, they were taken in a rotary shaker along with sodium hypochlorite and water (1:1) for 15-20 minutes and washed 3-5 times with distilled water. Then treated with absolute alcohol for 30 seconds and washed 3-5 times again with distilled water to remove alcohol and taken them to laminar air flow chamber for further treatments.

In the laminar-air-flow chamber suckers were treated with antioxidants like citric acid and ascorbic acid at different concentrations of 25, 50 and 75 mg/L for 15 and 30 minutes and washed with sterile water 3-4 times to remove the traces of them. Followed by this, a layer of leaf sheath has been removed and trimmed the base of explants and then treated with chemical sterilants like sodium hypochlorite at 10 and 20 ml in 150ml of sterile water for 5, 10 and 15 minutes along with one drop of tween 20 and washed 3-4 times using sterile

water. Another layer of leaf sheath was removed and trimmed the base to reduce the size of explants. Further, they were treated with mercuric chloride at 50, 100 and 150 mg in 150 ml of sterile water for 5 minutes and washed repeatedly 3-5 times with sterile water to remove traces of mercuric chloride effectively. Again removed a layer of leaf sheath and trimmed the base of explants. Furthermore, they were treated with an antibiotic streptocyclin (9% streptocyclin sulphate and 1% tetracycline hydrochloride) at 25, 50, 75 and 100 mg in 100ml of sterile water and washed immediately with sterile water. This is followed by removal of another layer of leaf sheath and trimmed the base to reduce the size ultimately to 1-2cm. Thus prepared shoot tip explant is later treated with L-Cystein HCL 50 mg in 1 litre of distilled water for a short period of time and washed repeatedly 3-5 times with sterile water before inoculation. Cystein serves as a very important indirect role of protecting cells from oxidative stress.

Media preparation

Murashige and Skoog (MS) medium was used as the basal medium. Sucrose at 3% (w/v) was added into the mixture. Growth regulators BAP (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) for shoot formation and IBA (0, 1.0, 1.5 and 2.0 mg/l) for rooting of banana were supplemented before pH adjustment. The pH of the medium adjusted to 5.6 with 0.1 M HCl and 0.1 M NaOH followed by addition of 0.8% (w/v) agar. A total of 30 ml of medium were poured into sterilized bottles and allowed for solidification. Then the bottles containing medium was autoclaved at 121ºC, 15 psi for 15 minutes and cooled before inoculation.

Culture initiation

The sterilised shoot tip explants were cultured on MS medium containing different concentrations of BAP and the cultured bottles kept in growth room at 25 ± 2^0 C under dark for one week and later transferred to a photoperiodic regime of 16:8 hours of light and dark cycle. With the aseptic cultures establishment (Plate 1), they were split into two halves and placed in the media bottles exposing the meristem region to medium. Later, regenerated shoots were sub cultured at 4-5 weeks intervals up to 5 cycles for multiplication using a modified media composition that contains 3.0 mg/l BAP, 25mg of adenine sulphate and 2-3ml of aonla juice. Observation on parameters like number of days taken for shoot initiation, shoot length, number of leaves/shoot and number of shoots were taken into consideration to study the explants regeneration capabilities.

Further, newly formed shoots were excised individually and transferred to rooting media consisting half strength MS salts and IBA (1, 1.5, 2 mg/l) along with activated charcoal. The observations on rooting behaviour of *in vitro* regenerated shoots were made after 4 week of culture. Completely randomized block design (CRD) was used for the analysis of data. The data were subjected to ANOVA by using software Wasp 1.0 developed by ICAR Research Complex, Goa (http://www.ccari.res.in/waspnew.html).

Results and Discussion

Effect of antioxidants on browning of explants

Within four weeks of culturing, the external leaf primordia of explants turned green. The size of the explants also increased, while blackening was observed at the base of the explant. This blackening may be due to secretion of phenolic compounds and no growth was observed. These undesirable exudates form the barrier round the tissue, preventing nutrient uptake and hindering growth therefore, in the beginning fresh shoot

tips were transferred to new media every two weeks.

The results on influence of antioxidants citric acid and ascorbic acids on browning of explants are presented in the Table 1. The level of browning was found maximum with the control, without any kind of treatments (++++) followed by explants treated with citric acid at 25 mg/l for 15 and 30 minutes (+++) and with ascorbic acid at 25 mg/l for15 minutes (+++). Interestingly, there was no browning when explant treated with both citric acid 50 mg/l and ascorbic acid 50 mg/l for a period of 30 minutes. The inhibition of browning might be due to activity of ascorbic acid to scavenge oxygen radicals produced when plant is wounded, therefore, protecting the cells from the damage resulting from the injury (Titov *et al*., 2006). Similar kind of opinions given by Ko *et al.,* (2009) in cavendish banana cv. Formosana and by Kariyana, *et al.,* (2013) in banana cv. Barnagn.

Influence of sterilants on contamination of explants

The results on influence of surface sterilant on contamination is presented in Table 2.It is evident from this table that per cent contamination of explants found highest with the use of sodium hypochlorite at 10 ml for 5 minutes (75%) and with the same sterilant treated for 10 minutes (62%). Sodium hypochlorite is a potential oxidizing agent containing an active agent of free hypochlorous acid. The inhibition of growth and development of microorganisms by hypochlorite was due to the penetration of the germicide into the cell leading to interference with the cytoplasmic metabolism.

Within the mercuric chloride levels tried, highest per cent contamination was found with 50 mg for 2 minutes (56%) and lowest was recorded with 150 mg for 5 minutes (36%). This could be attributed to the germicidal action of heavy metal ion mercury at higher concentration against fungi and bacteria. Mercury combines with the compounds containing sulfhydryl radical causing inactivation of metabolism of the organism.

For controlling bacterial infection streptocyclin was also used. Individually, streptocyclin at 25 mg for 2 minutes showed highest per cent contamination (60%) while; the same sterilant at75 mg for 2 minutes recorded the lowest per cent contamination (30%). However, when all these best treatments were used in sequence one after the other for a known period of time the contamination was drastically reduced. It was lesser in sodium hypochlorite at 20 ml for 15 minutes + mercuric chloride at 150mg for 5 minutes + streptocyclin at 75 mg for 2 minutes (15%).

Many decontamination protocols exist. They differ in explant type and size, disinfection procedure (single or double sterilisation) (Hamill, *et al*., 1993), type of disinfectant and its concentration and treatment duration (Wong, 1986).L-Cysteine HCl was also used after all the above sterilants at a concentration of 50mg/l. Cysteine serves a very important indirect role of protecting cells from oxidative stress. It is the rate limiting amino acid used in the synthesis of the tri-peptide glutathione. Glutathione has the ability to oxidize dehydroascorbic acid to ascorbic acid, which is the primary aqueous antioxidant involved in blocking lipid peroxidation. It is also the substrate for selenoprotein antioxidation enzymes. Glutathione is rapidly depleted and cells will die in the absence of L-cysteine or cysteine equivalents and therefore, it needs to be supplemented.

Shoot initiation and multiplication

The concentration and combination of auxin

and cytokinin in the nutrient medium is the key factor which determines successful plant regeneration (Razdan, 1993).The differences in the treatments with respect to number of days required for shoot initiation from the date of inoculation, length of shoot (cm) and number of leaves per shoot (Table 3) highlight the importance of exogenously supplied growth regulators to achieve higher multiplication rates. MS media supplemented with BAP 3.0 mg/l considered to be the optimum level for early and better induction of shoots in banana.

There was no shoot initiation with MS media supplemented up to1.0 mg/l BAP apart from the control. The data also revealed number of days required for shoot production after establishment of cultures was significantly affected in the presence of plant growth regulators. The minimum number of days required (16.66 days) for the response of shoot initiation was observed in the treatment MS media containing 3.0 mg/l of BAP, which was followed by 2.5 mg/l BAP (20.33 days). However, maximum time required (25.33 days) for shoot induction was noticed with MS media supplemented with 1.5 mg/l BAP. This may be due to insufficient endogeneous and exogenous supply of plant growth hormones to the explant tissue. These results are in conformity with the work of Kalimuthu *et al.,* (2007) when inoculated banana shoot tip explants on MS medium with six different combinations of BAP and NAA, after few days the explants swell and turn green and produce shoots within four weeks.

The observations recorded on number of leaves also varied significantly with different concentrations of BAP. When there was no shoot formation at lower concentration of BAP and with basal media composition, it was obvious no leaves could be expected. However, variations in hormonal concentrations registered significant differences for the number of leaves produced per shoot. The number of leaves per shoot observed at 30 days after inoculation was highest with the treatment containing BAP at 3.0 mg/l (2.43), followed by BAP at 2.5 mg/l (1.83). As anticipated, lesser number of leaves per shoot was recorded with MS supplemented with 1.5 mg/l BAP (1.36). These results are in line with that of Rahman *et al*., (2004) where he could produce maximum of 3.12 leaves /plantlets at 30 days of inoculation with 5.0 mg/l BAP in banana cv. BARI-1. Aman *et al.,* (2018) also registered maximum number of leaves per shoot was produced on the medium supplemented with BAP 4 mg/l.

BAP also had significant effect on length of shoots at 30 days of inoculation. Higher concentration of BAP showed good response compared to lower concentrations (Plate 2A). MS supplemented with BAP 3.0 mg/l proved most effective in which shoot length was found to be maximum (2.13 cm) and was significantly superior than all other treatments, followed by 2.5 mg/l BAP (1.63 cm). After 60 days of inoculation, shoots grew significantly longer with BAP at 3.0 mg/l (5.33 cm) compared to rest of the treatments. Next to this, BAP 2.5 mg/l (5.06 cm) registered longer shoots. For shoot multiplication, media composition was modified with the addition of adenine sulphate and aonla juice. When the media supplemented with BAP (3 mg/l) + Adenine sulphate (25 mg/l) + Aonla juice (2-3ml), highest shoot length (2.40 cm), number of leaves (2.82) and number of shoots (7.40) (Plate 2B) was registered (Table 4).

In banana, BA is the preferred cytokinin and is usually added in the concentration of 0.1-20mg/l (Banerjee and Langhe 1985). It has been observed that banana multiplication rate is genotypic dependent as well as variable behavior has been observed among cultures

initiated from same banana genotypes cultured *in vitro* (Israeli *et al.*, 1995; Mendes *et al.*,1996), the differences in growth rate may be due to physiological response of different rhizomes. The effectiveness of BAP over other cytokinins in inducing multiplication of shoot tip cultures has been reported in different cultivars of banana (Buah *et al.*, 2010; Farahani *et al*., 2008; Rahman *et al*., 2006).

A maximum of three multiple shoots per explant were observed in explants cultured on MS medium supplemented with 3.0 mg/l BAP and 0.2 mg/l IAA (Kalimuthu *et al.*, 2007). The results are also in agreement with the findings of (Rahman *et al.*, 2013) where best regeneration of shoots was observed in MS medium containing 4 mg/l BAP in banana cv. Agnishwar. The maximum per cent regeneration was observed with BAP 4 mg/l in both the subcultures of banana cv. Rajapuri (Aman *et al.*, 2018). Superiority of BAP may be due to the fact that it has a marked effect in stimulating the growth of auxillary and adventitious buds and foliar development of shoot tip cultures.

The frequency of bud formation doubled in media with BAP at 5 mg/l compared to media supplemented with 3 mg/l BAP and it was greater with BAP at 7 mg/l (Bhosle *et al*., 2011). If the production of highly proliferating meristem cultures is required, a tenfold higher concentration of BA may be used in the culture medium (p4 medium containing 22.5mg/l BA and 0.175mg/l IAA). However, higher concentration of the cytokinin BA tends to have an adverse effect on the multiplication rate and morphology of the culture and should therefore be avoided (Strosse *et al*., 2015).

Table.1 Effect of antioxidants on browning of explants

Sl.No.	Antioxidants	Level of browning
1	Control	++++
2	Citric acid 25mg/l 15 min	+++
3	Citric acid 50mg/l 15 min	++
4	Citric acid 75mg/l 15 min	+
5	Citric acid 25mg/l 30 min	+++
6	Citric acid 50mg/l 30 min	+
7	Citric acid 75mg/l 30 min	+
8	Ascorbic acid 25mg/l 15 min	+++
9	Ascorbic acid 50mg/l 15 min	++
10	Ascorbic acid 75mg/l 15 min	+
11	Ascorbic acid 25mg/l 30 min	++
12	Ascorbic acid 50mg/l 30 min	+
13	Ascorbic acid 75mg/l 30 min	+
14	Citric acid 50mg/l 30 min + Ascorbic acid 50mg/l 30 min	-

Table.2 Influence of sterilants on contamination of explants

Sl No.	Sterilant	Per cent Contamination (%)
1	NaOCl 10 mlfor 5 min	75
2	NaOCl 10 ml for 10 min	62
3	NaOCl 10 ml for 15 min	54
4	NaOCl 20 ml for 5 min	64
5	NaOCl 20 ml for 10 min	58
6	NaOCl 20 ml for 15min	52
7	$HgCl_2$ 50mgfor 2 min	56
8	$HgCl_2$ 100 mg for 2 min	44
9	$HgCl_2$ 150 mg for 2 min	40
10	$HgCl_2$ 50mg for 5 min	52
11	$HgCl_2$ 100 mg for 5 min	40
12	$HgCl_2$ 150 mg for 5 min	36
13	Streptocyclin 25 mg for 2 min	60
14	Streptocyclin 50 mg for 2 min	43
15	Streptocyclin 75 mg for 2 min	30
16	NaOCl 20 ml, 15 min + $HgCl_2$ 150 mg, 5 min + Streptocyclin75 mg, 2 min	15

Table.3 Effect of BAP on shoot initiation and proliferation

Treatments BAP(mg/l)	No. of days taken for shoot initiation	30 days after inoculation		60 days after inoculation	
		Shoot length (cm)	No. of leaves / shoot	Shoot length (cm)	No. of leaves / shoot
Control	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00
1.0	0.00	0.00	0.00	0.00	0.00
1.5	25.33	1.16	1.36	4.00	2.23
2.0	21.66	1.46	1.56	4.16	2.53
2.5	20.33	1.63	1.83	5.06	3.06
3.0	16.66	2.13	2.43	5.33	3.43
S. Em. ±	0.146	0.014	0.014	0.010	0.010
C.D. @ 1%	1.061	0.106	0.106	0.075	0.092

Table.4 Modified media composition for shoot multiplication

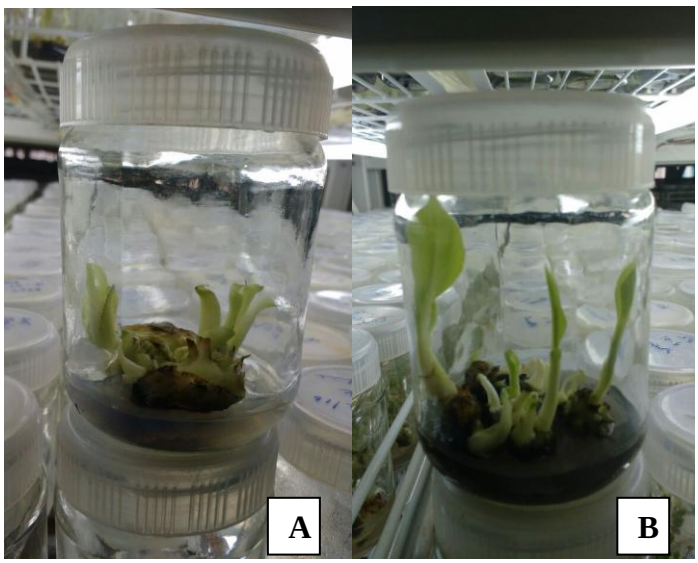
Treatments	Shoot length (cm)	No. of leaves	No. of shoots
BAP (3 mg/l) + Adenine sulphate (10 mg/l)	2.18	2.42	3.40
BAP (3 mg/l) + Adenine sulphate (25 mg/l)	2.30	2.50	4.40
BAP (3 mg/l) + Adenine sulphate (10 mg/l) + Aonla juice (2-3ml)	2.34	2.70	5.40
BAP (3 mg/l) + Adenine sulphate (25 mg/l) + Aonla juice (2-3ml)	2.40	2.820	7.40
S. Em. ±	0.006	0.018	0.109
C.D. @ 1%	0.046	0.170	1.012

Table.5 Effect of IBA on rooting at 4 weeks after inoculation

Treatments IBA (mg/l)	No. of days for rooting	Root length (cm)	No. of roots per explant
Control	0.00	0.00	-
1.00	21.28	1.51	*
1.50	19.60	2.59	**
2.00	16.50	3.01	***
S. Em. ±	0.042	0.006	
C.D. @ 1%	0.394	0.051	

Plate.1 Establishment of contamination free cultures

Plate.2 Shoot initiation (A) and multiplication (B)



Root initiation

Induction of healthy root system from the regenerated shoots is an essential part for successful development of plantlets. Banana shoots obtained from multiplication process were further cultured in MS basal medium supplemented with different concentrations of IBA (1.0, 1.5 and 2.0 mg/l) along with control for root induction. IBA exerted significant effects on number of days taken for root initiation, number of roots per regenerated shoot, root length (cm) and number of roots when cultured in MS media containing different concentrations of IBA (Table 5).The data was recorded at four weeks after inoculation and reveals that IBA (2.0 mg/l) when added in MS media proved superior over other treatments in almost all rooting parameters.

The number of days taken for root initiation ranged between 16.50 and 21.28 days. Early root initiation was observed (16.50 days) with IBA treatment at 2.0 mg/l which was significantly higher to other recorded values, followed by IBA at 1.50 mg/l (19.60). There was no root initiation in the basal media without any supplement of growth regulator. Root elongation was increased at all levels of auxin used. IBA at 2.0 mg/l proved to be most effective to increase the length of roots with maximum of 3.01 cm followed by IBA at 1.5 mg/l (2.59 cm). Number of roots produces was also higher with IBA at 2.0 mg/l (***), while lesser number of roots was noticed in MS media supplemented with 1.0 mg/l IBA (*).

It was found that auxins promoted root initiation and growth by inducing the cells to the pericycle and parenchyma to dedifferentiate and start initial cell division process (Celenza *et al.,* 1995).Accumulation of auxin within the root tissues may cause an increase in the number of adventitious root formation (Laskowski *et al.,* 1995). An increase in myelin basic protein (MBP) kinase activity in response to auxin treatment which provides a stimulus for mitogen activated protein kinase (MAPK) activation and initiated mitotic process which induces

dedifferentiation of xylematic or parenchyma cells that acquired meristematic activity resulting in cell division and increased number of adventitious roots (Mockaitis and Howell, 2000). IAA, NAA or IBA are the growth hormones commonly included in the medium concentrations between 0.1 and 2 mg/l. For some genotypes (*Musa* spp. ABB and BB group) that produce compact proliferating masses of buds, activated charcoal (0.1-0.25%) added to the regeneration/ rooting medium enhanced shoot elongation and rooting (Strosse *et al*., 2015).

It may be concluded from this experiment that citric acid and ascorbic acid were good antioxidants in reducing phenol exudation from the explants thus prevents browning. All the chemical sterilants effective in reducing contamination from microbial infection when they were used in sequence one after the other. MS media supplemented with BAPat 3 mg/l was the best hormonal concentration for shoot initiation and BAP at 3 mg/l and adenine sulphate at 25 mg/l with 2-3 ml of Aonla juice for shoot multiplication. MS media at half strength with IBA at 2 mg/l was effective in better roots formation in presence of activated charcoal.

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