academicJournals

Vol. 15(11), pp. 401-407, 16 March, 2016
DOI: 10.5897/AJB2015.14883
Article Number: F200ADD57542
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

A commercial micropropagation protocol for virupakshi (AAB) banana via apical meristem

Karule P., Dalvi V., Kadu A., Chaudhari R., Subramaniam V. R.*, and Patil A. B.

Biotech Laboratory, Jain R&D, Agri Park, Jain Hills, Shirsoli Road, Jalgaon, 425001, India.

Received 24 July, 2015; Accepted 19 January, 2016

In vitro **micropropagation of banana (*Musa* spp.) cv.virupakshi (Hillbanana) was studied. Suckers were collected from the germ plasm block of Jain R&D (originally established from the suckers from Palani Hills, Tamil Nadu) during summer. The sucker surface sterilized with 1% NaOCl for 30 min gave 100% survival without any contamination. Apical meristems that were isolated and cultured on MS based media supplemented with benzylaminopurine (BAP) 10.0 mg/l and IAA1.0 mg/l gave higher number of shoots (134.3 shoots/explant) within168 days (24 weeks). Kinetin 2.0 mg/l and NAA0.5 mg/l gave early rooting in just five days with 6.6 roots per plant. Observations were recorded after every four weeks up to six sub-culturing. Acclimatization was done in poly house, followed by shade house under 50% light conditions. The hardened plants when shifted to the field showed luxurious growth. The regenerated micro propagated banana plants were tested for genetic uniformity through 13 inter simple sequence repeat (ISSR) markers recommended by NCS-TCP, DBT. Profiles obtained by all the three ISSR primers namely, 834, 840 and 850, respectively exhibited similar banding patterns, which revealed the existence of genetic uniformity in micro- propagated plants.**

Key words: Micropropagation, Virupakshi, hill banana, banana bunchy top virus.

INTRODUCTION

Banana is one of the most economically important fruit crops that can be produced in almost all parts of India as a vital source of energy. Plantains and bananas (*Musa* spp.) constitute staple food for rural and urban consumers in the humid tropics and are the fourth most important global food commodity after rice, wheat and milk. Annual production of *Musa* spp. in the world is about 101.99 million tons (FAO, 2012).

India is the largest producer and consumer of banana with many varieties under cultivation. Among the cultivars that are grown and consumed in specific areas and purposes, hill banana is one amongst them. Hill bananas (AAB genome) have two eco types namely Virupakshi and Sirumalai, known for their special flavor, long shelf life and are unique to some pockets of Tamil Nadu, India. This variety is grown in limited area with traditional planting material and cultivation methods without much scientific intervention. This has resulted in infection and

*Corresponding author. E-mail: dr.subramaniam@jains.com. Tel: +912572260011.

accumulation of several diseases in plantations, the most important among them being banana bunchy top virus (BBTV). This has reduced the productivity, quality and profitability for the farmers.

BBTV has been the sole cause for reduction in hill banana cultivation from 18, 000 ha in 1970s to about 2,000 ha at present (Elayabalan, 2010). BBTV is transmitted by the aphid *Pentalonia nigronervosa* (Hu et al., 1996). Availability of virus free planting material may help in restoring the area, productivity and income. Micro propagation is an established method to multiply virus free planting material in banana. *In vitro* micro propagation has been widely adopted as an alternative means for production of disease free banana planting material. There are many reports on micro propagation of other banana varieties (Sadik et al., 2012) using shoot tip, but none or very limited on hill banana.

Recently, *in vitro* micro propagation technique for 'Virupakshi' through embryonic cell suspension culture has been reported. However, the method needs expertise for developing suspension cultures. Keeping these facts in view, in the present research investigation, the study has successfully developed a commercially viable micro propagation protocol for hill banana.

MATERIALS AND METHODS

Micro propagation

The research investigation was conducted at Tissue Culture Laboratory of Jain R&D, Jain Irrigation Systems Limited, Jalgaon (Maharashtra) India. Sword suckers were collected from virus free and true to type Hill banana plants cv. Virupakshi from the germ plasm block of Jain R&D farm. Before sucker collection, the mother plants were indexed for viruses (BBTV and CMV) to ensure that they are free from viruses. The suckers were brought to laboratory and cleaned under running tap water for 30 min. Following this, two to four leaf sheaths were carefully removed, and explants were trimmed into 50 x 30 mm by cutting the top and base. The explants were surface sterilized using 1% Ridomil and two drops of Tween-20 for 30 min.

Further, explants were surface sterilized using 1% NaOCl for 30 min followed by four to five washings with sterile reverse osmosis water four times each for 5 min. Excised shoot tips (10 X 5 mm) were inoculated vertically in 50 ml of modified Murashige and Skoog medium (MS) media (Murashige and Skoog, 1962) (basic salts plus thiamine HCl 2.0 mg/l, nicotinic acid 0.5 mg/l, pyrodoxine HCl 0.5 mg/l, meso-inositol 10 mg/l, L-tyrosine10 mg/l) supplemented with IAA (0.5 to 1.0 mg/l), benzylaminopurine (BAP) (0.0 to 20 mg/l), agar 0.5% (w/v) and sucrose 3% (w/v). The pH of medium was adjusted at 5.8±0.02 before auto claving at 15 psi for 15 min. All cultures were incubated at 25±2°C and were exposed to a photoperiod of 16/8 h light and dark cycling under 3000 lux density provided by white cool fluorescent tubes (40 W, Philips, India) with 70±5% relative humidity maintained in the culture room. Ten (10) explants were taken for each treatment and replicated thrice.

After initiation, explants were transferred into multiplication medium (MS+BAP+IAA, Table 3). Sub-culturing was carried out after every four weeks. In the first sub culturing, shoot tip was cut into two equal halves and transferred into multiplication medium. In further sub culturing, explants were cut into two equal halves and inoculated into multiplication medium, this process was repeated up to six sub-culturing.

After last sub culturing, 3 to 4 cm long shoots were detached from clumps and transferred to the rooting medium supplemented with various concentrations of kinetin (0.0 to 2.0 mg/l) and NAA (0.0 to 1.0 mg/l). Observations were recorded on time taken for root initiation, number of roots, root length and root weight after 4 weeks. Rooted plantlets were removed from glass bottles and washed under running tap water to remove culture media from plantlets because sucrose in agar encourages growth of microorganisms. After washing the plantlets, roots were dipped in fungicide solution (0.1% Ridomil) and transferred to 20 cc plant tray containing peat moss medium for primary hardening. Initially, plant trays were transferred into poly tunnel for 18 days (24 to 26°C, 80 to 90% RH and 7000 lux natural light). After that, plants were transferred to green house for 10 days (26±2°C, 70 to 80% RH and 10000 to 15000 lux natural light). Primary hardened plants were finally transferred in 1.2 L bags having press mud cake, silt, coconut husk and farm yard manure (1:1:1:1) under 50% shade net house for 45 days at 40% RH. Secondary hardened plants were ready for field plantation.

Table 1. Primers used for testing of genetic fidelity of micro propagated plants.

Primer	Sequence
UBC 807	GA GAG AGA GAG AGA GT
UBC 808	AGA GAG AGA GAG AGA GC
UBC 811	GAG AGA GAG AGA GAG AC
UBC 812	GAG AGA GAG AGA GAG AA
UBC 818	CAC ACA CAC ACA CAC AG
UBC 830	TGT GTG TGT GTG TGT GG
UBC 834	AGA GAG AGA GAG AGA GYT
UBC 836	AGA GAG AGA GAG AGA GYA
UBC 840	GAG AGA GAG AGA GAG AYT
UBC 841	GAG AGA GAG AGA GAG AYC
UBC 842	GAG AGA GAG AGA GAG AYG
UBC 850	GTG TGT GTG TGT GTG TYC
UBC 868	GAA GAAGAAGAAGAAGAA

Genetic fidelity testing

Total genomic DNA of micropropagated plants was isolated using two gram fresh leaf from 13 micro propagated plants and mother plant using CTAB method (Dellaporta et al. 1983). The DNA pellet was dissolved in Tris EDTA (TE) buffer (200 µl) and quantified on nano drop spectrophotometer. DNA was stored at -20°C. Working stock of genomic DNA was diluted to 50 ng/µl concentration. Polymerase chain reaction (PCR) was performed using different inter simple sequence repeat (ISSR) primers (Table 1) suggested for banana by NCS-TCP, DBT (Anonymous, 2008). A total of 25 µl PCR reaction was performed using 10 mM Tris-HCl , pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 1.0 µM primer, 100 ng genomic DNA and 1 unit of *Taq* DNA polymerase. Amplification was performed infollowing steps: 5 min at 94°C, [45 s at 94°C, 45 s annealing temperatures specified for each primer and 2 min extension at 72°C] x 35 cycles with final extension for 7 min at 72°C. The gel electrophoresis was carried out using a submarine horizontal gel assembly in 1.5% agarose gel with ethidium bromide. The PCR product was visualized in a gel documentation system (Alpha Innotech Corporation, USA).

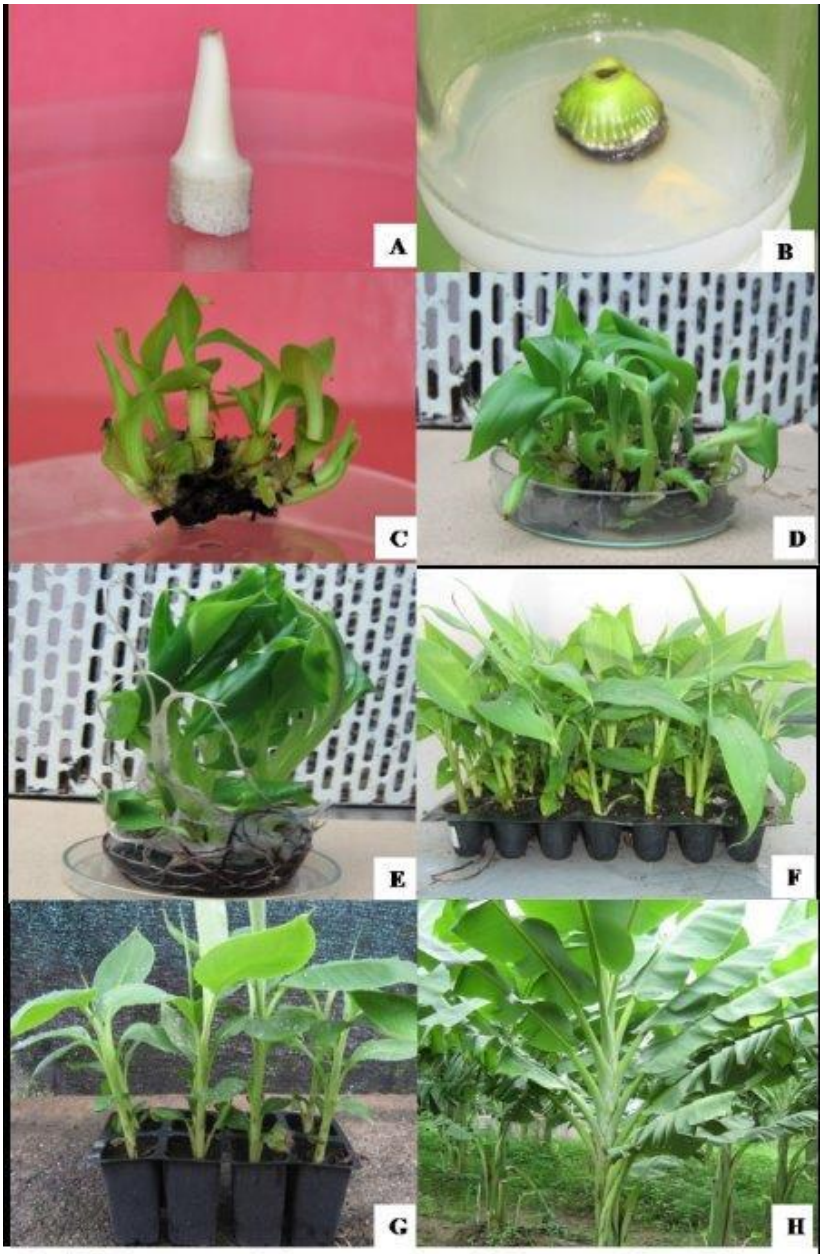

Figure 1. Various stages of micro propagation of hill banana cv. Virupakshi. (A) Trimmed sucker (*Ex* plant), **(B)** initial apical meristem, **(C)** shoot multiplication, **(D)** elongation, **(E)** rooting, **(F)** primary hardened plants, **(G)** secondary hardened plants, and **(H)** micro propagation plants in field.

RESULTS AND DISCUSSION

In vitro culture initiation and multiplication

Among the different concentrations of BAP and IAA, modified MS+BAP10.0 mg/l+IAA1.0 mg/l was found to be the best for initiation and multiplication (Figure 1A to C, Table 2). This combination produced 134.33 shoots per sucker and 2 cm long plantlets followed by modified MS+BAP10.0 mg/l+IAA0.5 mg/l, which produced127.66 plantlets/sucker with average plant height (1.8 cm) after 6th sub culturing. It was observed that when the concentration of BAP was further increased up to 20 mg/l along with IAA0.5 mg/l, the treatment caused an adverse effect on proliferation and the number of plantlets reduced to 57.33% (Table 2). It appears that high concentration of cytokine is detrimental to shoot proliferation in banana plants. Similar observations were

Table 2. Effect of phytohormones on shoot multiplication.

Treatment (mg/l)	Average no. of explants survived after sub culturing				Average plant height (cm)
	3rd	4th	5th	6th	
Control	8.0	10.33	13.66	17.66	3.33
BAP5.0+IAA0.5	9.0	18.33	33.33	60.33	2.67
BAP10.0+IAA0.5	10.33	23.33	54.33	127.66	1.8
BAP15.0+IAA0.5	9.66	21.66	46.00	87.99	1.73
BAP20.0+IAA0.5	7.33	14.33	28.66	57.33	1.67
BAP5.0+IAA1.0	9.66	19.66	38.33	86.66	2.33
BAP10.0+IAA1.0	10.33	23.33	56.00	134.33	2.00
BAP15.0+IAA1.0	8.66	18.99	40.00	100.00	1.83
BAP20.0+IAA1.0	8.33	17.66	33.33	70.33	1.67
CD	1.450	2.051	2.703	5.724	0.788
SEM±	0.484	0.685	0.903	1.912	0.263

Table 3. Effect of phytohormones on rooting.

Treatment (mg/l)	Days taken for root induction	No. of roots	Root length (cm)	Root weight (g)
Control	10.0	3.0	5.4	0.0374
Kinetin 0.5+NAA0.5	7.3	4.0	6.0	0.0391
Kinetin 1.0+NAA0.5	6.3	4.3	6.9	0.0418
Kinetin 1.5+NAA0.5	5.6	5.3	7.0	0.0425
Kinetin 2.0+NAA0.5	5.0	5.6	12.6	0.0734
Kinetin 0.5+NAA1.0	7.3	5.0	5.76	0.0332
Kinetin 1.0+NAA1.0	7.0	5.3	6.4	0.0399
Kinetin 1.5+NAA1.0	5.6	5.6	9.4	0.0556
Kinetin 2.0+NAA1.0	5.0	6.6	15.3	0.0908
CD	0.744	0.742	1.459	0.002
SEM±	0.248	0.248	0.487	0.001

also reported by Vuylsteke (1989) and Arinaitwe et al. (2000) during multiplication of 'Nzizi', 'Kibuzi' and 'Ndiziwemiti', respectively.

Rahman et al. *(*2004) recorded highest number of leaves per shoot at 30 days interval with 5 mg/lit BAP which was at par with BAP4 mg/l +NAA 1.5 mg/l. Higher concentrations of BAP and kinetin beyond optimum levels were also reported to cause necrosis and reduction in shoot formation during *in vitro* multiplication of Nendran (Rabbani et al., 1996). Other experiments observed highest number of shoots per explants at 28 days (3.11) with 5.0 mg/l of BAP and kinetin (Damasco and Barba, 1984). BAP alone 10.0 mg/l gives 10.10 shoots per ex plant (Gupta, 1986) with BAP and higher proliferation rate with increase in number of cycles (first cycle 11.32 and 4th cycle 17.78 number of shoots) was also observed.

In vitro rooting

The data presented in Table 3 clearly revealed kinetin 2.0 mg/l+NAA1. 0 mg/l gave early rooting (Figure 1E) in just five days, with higher number of roots (6.6 roots/plantlet) and root length (15.3 cm) in four weeks. The next best combination was kinetin 2.0 mg/l+NAA0.5 mg/l which induced lower number of roots (5.6) and root length (12.6 cm) in four weeks; while, lowest number of root (3.0) and root length (5.4 cm) was observed in control. It was observed that when cultures were shifted to lower concentrations of kinetin (1-2 mg/l) and auxins (NAA 0.5 to 1.0 mg/l) the number of roots and root length were increased while the time taken for root initiation had decreased. Low cytokinin of concentrations with lower cytokine into auxin ratio is useful for root initiation (Wong, 1986).

Naphthalene acetic acid (NAA) was found to be effective at very low concentrations for root initiation of banana. According to Cronauer and Krikorian (Cronauer and Krikorian, 1984). NAA 1.0 mg/l is suitable for root initiation in Musa *sp*. Arinaitwe et al. (2000) observed rooting on MS medium containing NAA 1.2 µM during the study of multiplication rate effects of cytokinins on Kibuzi, Bwara and Ndizwemit banana cultivars. Pruski et al. (2005) also reported best rooting response in a

Table 4. Comparative growth of plantlets during primary and secondary hardening.

S/N	Primary hardening				Secondary hardening			
	Plant height (cm)	No. of leaves	No. of roots	Root weight (g)	Plant height (cm)	No.of leaves	No. of roots	Root weight (g)
1	8.80	5.66	4.33	1.23	13.73	5.00	6.33	7.09
2	9.50	5.66	5.83	1.34	14.73	5.33	7.33	7.10
3	8.8	4.66	7.66	1.32	15.46	5.66	6.00	7.04
4	8.73	7.16	6.66	1.63	16.13	6.00	6.33	8.68
5	9.20	5.66	7.16	1.40	17.03	6.33	7.33	7.10
6	9.30	6.66	6.33	1.32	15.00	6.66	6.66	8.04
7	8.70	7.33	8.00	1.51	16.26	7.33	7.66	8.76
8	9.70	7.16	7.33	1.43	15.76	6.66	7.66	7.37
9	8.70	6.33	5.66	1.13	16.50	7.00	6.33	8.09
10	9.50	6.00	6.66	1.52	17.00	5.33	6.66	7.98
CD	0.722	1.581	1.715	0.247	1.957	0.990	1.085	0.766
SEM±	0.243	0.532	0.577	0.083	0.659	0.333	0.365	0.258

combination of IBA and NAA. However, De Langhe (1985) and Novak et al. (1990) used half strength MS+1.0 mg/l IBA, whereas Cronauer and Krikorian (1984) used auxin-free MS for rooting of banana microshoots. On the other hand, Banerjee and De Lahange (1986) and Azad and Amin (2001) obtained rooted banana shoots in half strength MS medium supplemented with 0.2 mg/l IBA. Akbar and Roy (2006) reported 1.0 mg/l IBA for best rooting response of *in vitro* cultured plants.

Hardening of *in vitro* raised plant

It is generally seen that maximum mortality of micropropagated plants occurs during acclimatization phase because plantlets undergo rapid and extreme changes in physiological functioning, histological and biochemical changes (Pati et al., 2013). To avoid this problem, it is necessary to harden the plantlets in two stages *viz.* primary and secondary hardening. During primary hardening (Figure 1F), plants showed hundred per cent survival and produced more number of leaves (6.3 leaves/plant), root (7.6 roots/plant) and root weight (1.63 g roots/plant) in the peat moss (Table 4). Problems of growth phase, primary hardening and secondary hardening need to be overcome for arriving at a commercially successful protocol of hardening (Anonymous, 2002). Vasane and Kothari (2006) reported that press mud cake mixed with soil was used as the optimal medium for producing sturdy plants during the secondary hardening (Figure 1G) process of banana cv. grand nain plantlets. Plants were showing luxurious growth after transfer into field (Figure 1H).

Genetic fidelity testing in regenerated plants

A protocol to be commercial must produce plants of true to type showing no genetic variation between the mother plant and the micro propagated plants. The Department of Biotechnology (DBT) has recommended a list of primer pairs that should yield identical banding patterns of the PCR products from mother plant and the micro propagated plants. Using 13 primers as recommended by DBT, the banding patterns of the mother plants and the micro propagated plants obtained from them, respectively were compared. Regenerated plants of hill banana cv. Virupakshi did not show any genetic variation between mother and micro propagated plants. Profiles obtained by three ISSR primers viz. 834, 840 and 850 as representing the patterns obtained are shown in Figure 2. As seen in these primers, all the 13 primers exhibited similar banding patterns for the mother plants and their micropropagated plants. These patterns reveal the existence of genetic uniformity in micro propagated plants.

Conclusion

Therefore, from the current research investigations, it is concluded that the study micro propagation protocol is successful for mass scale production of hill banana cv. virupakshi. This protocol can further be employed for the germplasm storage and production of disease free planting material.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

BAP, Benzylaminopurine; **ISSR,** inter simple sequence

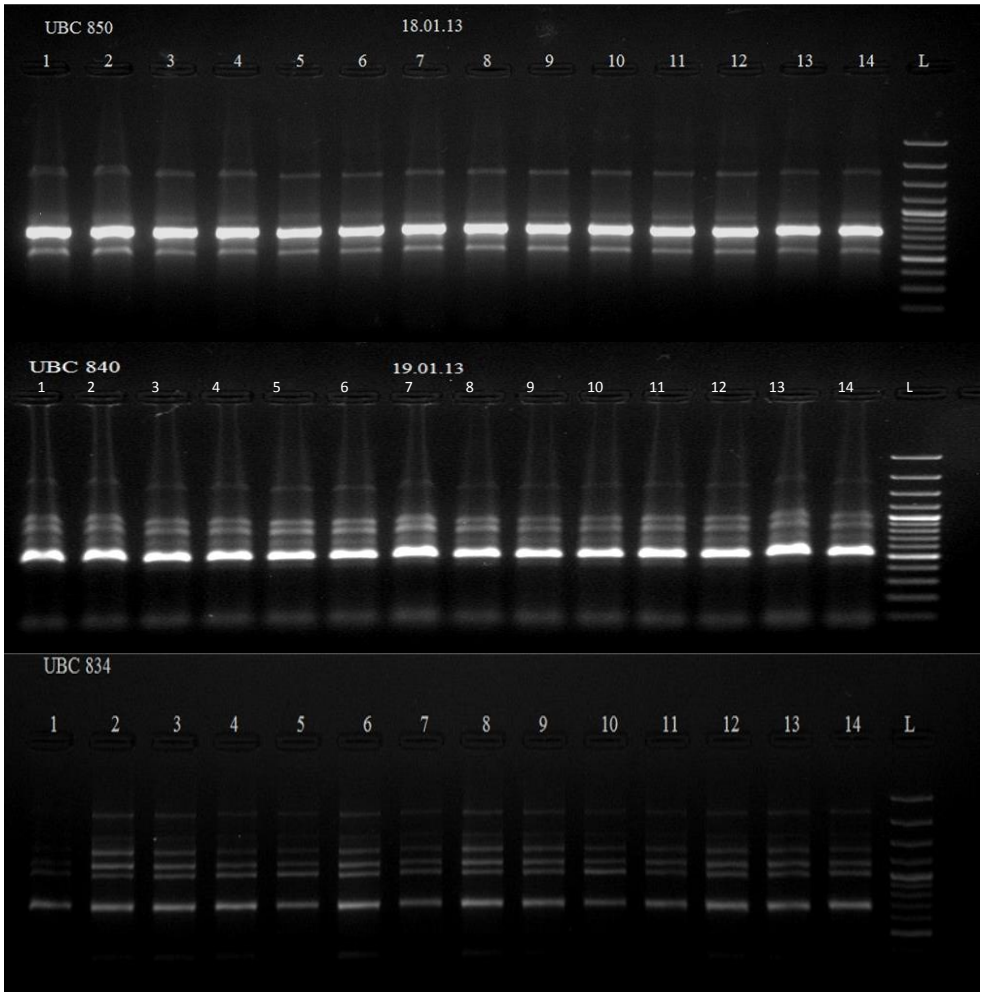

Figure 2. DNA finger printing pattern of mother plant (lane 1) and micro propagated plant (lanes 2 to 14) obtained by ISSR marker 834, 840 and 850. Lane L is DNA ladder.

repeat.

REFERENCES

Akbar MA, Roy SK (2006). Effects of liquid medium on rooting and acclimation of regenerated microshoots of banana (*Musa sapientum* L.) cv. Sagar. Plant Tissue Cult Biotechnol. 16:11-18.

Anonymous (2002). Hi-tech banana production practices, Jain Irrigation Systems Ltd. Jalgaon. pp. 1-28.

Anonymous (2008). Standard operating procedures for accredited test laboratory, National Certification System for Tissue Culture Raised Plants (NCS-TCP) Department of Biotechnology, Government of India, New Delhi, May 2008.

Arinaitwe G, Rubaihayo PR, Magambo MJS (2000). Proliferation rate effects of cytokinins on banana (*Musaspp.*) cultivars. SciHort. 86:13-21.

Azad MAK, Amin MN (2001). Rapid clonal propagation of banana (*Musa* sp.) using *in vitro* culture of floral bud apex. Plant Tissue Cult.11:1-9.

Banerjee N, De Langhe E (1986). A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (Banana and Plantain). Plant Cell Rep. 4:351-354.

Cronauer SS, Krikorian AD (1984). Rapid multiplication of banana and plantains by *in vitro* shoot tip culture. HortSci 19:234-235.

Damasco OP, Barba RC (1984). *In vitro* culture of Saba (*Musasp*.cv. Saba BBB). Philipp. Agric. 67:351-358.

De Langhe E (1985) Tissue culture technique for rapid clonal propagation and storage conditions of *Musa* (Banana and Plantain). Plant Cell Rep. 4:355-359.

Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA mini preparation: version II. Plant Mol. Biol. Rep. 1:19-21.

FAO, STAT (2012).http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor

Gupta PP (1986). Eradication of mosaic disease and rapid clonal multiplication of bananas and plantains through meristem tip culture. Plant Cell Tissue Organ. Cult. 6:33-39.

Elayabalan S, (2010). About Hill banana (AAB, Virupakshi and Sirumalai) and BBTV in Tamil Nadu, India. www.promusa.org/blogpost134.

Hu JS, Wang M, Sether D, Xie W, LeonhardtKW (1996). Use of polymerase chain reaction (PCR) to study transmission of banana bunchy top virus by the banana aphid (*Pentalonia nigronervosa*). Ann. Appl. Biol. 128:55-56.

Murashige T, Skoog FA (1962). Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Novak FJ, Afza R, Duren M, Omar MS (1990). Mutation induction by gamma irradiation of *in vitro* cultured shoot-tip of banana and plantain

(*Musa* cvs.). Trop. Agric. (Trinidad). 67:21-28.
Pati R, Mishra M, Chandra R, Muthukumar M (2013). Histological and biochemical changes in *Aegle marmelos* Corr. before and after acclimatization. Tree Genet. Mol. Breed. 3:1.
Pruski K, Astatkie T, Nowak J (2005). Tissue culture propagation of Mngolian cherry (*Prunusfruticosa*) and Nankingcherry (*Prunus tomentosa*). Plant Cell Tissue Organ. Cult. 82:207-211.
Rabbani MG, Au MH, Mondal MF (1996). Effect of BAP and IBA on micropropagation of some banana cultivars. Bangladesh Hortic. 25:47-52.
Rahman MZ, Nasiruddin KM, Amin MA, Islam MN (2004). *In vitro* response and shoot multiplication of banana with BAP and NAA. Am. J. Plant Sci. 3:406-409.
Sadik K, Arinaitwe G, Ssebuliba JM, Gibson P, Lugolobi C, Mukasa SB (2012). Proliferation and shoot recovery among the East African highland banana. Afr. Crop Sci. J. 20:67-76.
Vasane RS, Kothari RM (2006). Optimization of hardening process of banana plantlets (*Musa paradisiaca* L. var grand nain). Indian J. Biotechnol. 5:394-399.
Vuylsteke D (1989). Shoot tip culture for the propagation, conservation and exchange of Musa germ plasm (Practical manuals for handling crop germ plasm *in vitro* 2. International Board of Plant Genetic Resources, Rome, Italy).
Wong WC (1986). *Invitro* propagation of banana (*Musa* spp.): Initiation, proliferation and development of shoot-tip cultures on defined media. Plant Cell Tissue Organ. Cult. 6:159-166.