ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **29**(2): 175-184, 2019 (December)

PTC&B

Efficient Clonal Propagation of *Musa* sp. *paradisiaca* Cultivar Gopi and Clonal fidelity Assessment Using ISSR Marker

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Key words: Musa, Cultivar Gopi, Micropropagation, Clonal fidelity, ISSR marker

Abstract

Attempt was made towards optimizing an *in vitro* shoot multiplication of banana cultivar Gopi with subsequent assessment of genetic stability. Experiments on enhanced shoot multiplication were conducted in MS fortified with BAP, Kn and 2-iP (4 mg/l) for a period of 8 weeks. Administration of exogenous root inducing growth regulator was eliminated to reduce culture investment and economy. Treatment with BAP supplemented medium proved to be optimum for shoot multiplication whereas 2-iP produced satisfactory results for shoot elongation. Long term incubation in all the treatments had been favorable for developing efficient root system. Hardening of *in vitro* grown plantlets showed high rate of survival (95%) upon transfer to potted soil. Assessment of clonal fidelity through inter simple sequence repeats (ISSR) analysis revealed 100% uniformity.

Introduction

Banana is a major fruit crop with multidimensional roles ranking as the fourth most crucial global food commodity in terms of gross production (FAO 2001, INIBAP 1992). As an important food and fruit crop, banana not only potentially contributes to the food security of a large number of people all over the world. It has also a definite role in uplifting and affecting the economic status of innumerable rural small holders in agricultural countries like India (Mahalakshmi et al. 2016). Moreover, the banana plant

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DOI: https://doi.org/10.3329/ptcb.v29i2.44506

has been inevitably holding a sacred position in Indian cultural heritage from the ancient times. India is known to be one of the centers of origin of *Musa* (Stover and Simmonds 1987) where north-eastern states cover an astounding diverse natural *Musa* population.

Among them, Tripura is an exclusive address to numerous cultivated bananas like Sobri, Chapa, Kaanch kola and Gopi which have been under local cultivation practices by small scale farmers since long time. *Musa* × *paradisiaca* L. cultivar Gopi is a well-known cultivar which has certainly developed as a good commercial banana among the people of this region owing to its better yield potential, sweet taste and utilities in holy purposes. But the plantation and cultivation programmes of this particular cultivar in this region are inadequate to meet the ever increasing demands of the local people and also its developing export potential. In addition, large scale production, maintenance of existing banana plantation is challenged by numerous limiting factors like high susceptibility to pathogens (Persley and De Langhe 1987), poor suckering ability (Ndubizu 1985), pests and diseases, poor agronomic practices and post-harvest constraints (Robinson 1996). Under the circumstances, *in vitro* micropropagation may prove to be dependable to overcome the limiting constraints for commercial mass propagation of disease free and true to type planting material of *Musa* × *paradisiaca* cultivar Gopi. Since tissue culture method is a sophisticated technique, it does not always confirm to be economically viable (Tomar et al. 2007). So, a necessary approach in the possible minimization of culture requirements becomes imperative for cost effective, cheap, fast and simple technique of optimum healthy banana production. Two important criteria of successful *in vitro* rapid propagation of banana are efficient development of multiple shoots and generation of genetically homogenous and uniform plantlets. Cytokinins have been known to induce high frequency *in vitro* shoot regeneration and multiplication in banana (Buah et al. 2000 and 2010). Application of different plant growth regulators during the culture and changes in habituations are known to be associated with genetic instability of the plants. Therefore, identification of somaclonal variations is regarded as important steps for quality control in tissue culture produced plant (Soniya et al. 2001). Various methods can be used to detect the genetic stability of regenerated plant but the most reliable method is to use molecular markers. Molecular markers are considered as a suitable method to detect the genetic variability among the plants (Zerihum et al. 2009). Among the molecular markers, Inter Simple Sequence Repeats (ISSR) marker is considered as dominant markers (Tsumura et al. 1996). It is cost effective and highly reliable method in genetic variability study (Borba et al. 2005). Study on somaclonal variations of banana using ISSR markers has also been reported by several researchers (Rout et al. 2009, Ying et al. 2011, Choudhury et al. 2015, Nandhakumar et al. 2017). The present study is an attempt towards optimizing a suitable *in vitro* mass multiplication method of *Musa* cultivar Gopi emphasizing on the reduction of culture needs like use of growth regulators and subculture phases and also subsequent assessment of clonal fidelity with ISSR markers.

Materials and Methods

An *in vitro* clonal propagation protocol has been established for the *Musa* sp. cultivar Gopi through multiple shoot induction focusing on sterilization steps and explant preparation following Sinha et al. 2018. To ensure efficient *in vitro* mass propagation of Gopi using long term subculture phase minimizing the application of growth regulator and also to assess the clonal fidelity of the regenerants using ISSR markers, present study was conducted.

Mass propagation of clones and maintenance of banana cultivar Gopi was achieved in prolonged subculture phase in MS with BAP, Kn and 2-iP. Well-developed shoot clusters were subcultured into fresh MS supplemented with 4 mg/l BAP, Kn and 2-iP and kept in prolonged incubation of culture for 8 weeks without any further intervening subculture. Observation on number of shoots per cluster, number of leaves per shoot, number of roots per shoot, shoot size (cm), leaf size including length (cm) and width (cm) and root size (cm) were recorded after 8 weeks. A mean of five replicates of shoot clusters were used in each treatment. Root inducing growth regulator was not applied in the subsequent stages to simplify the culture needs and produce a possible cost effective method. After 8 weeks of culture, the elongated shoots with well-developed root system were detached carefully from the base of the shoot cluster with a sterilized scalpel and thoroughly washed with water. The regenerated clones were finally placed in tap water *ex vitro* for a few days. This was followed by transferring the *in vitro* grown plantlets to potted soil mix (Soil : sand : soilrite = 2 : 1 : 1) in the shade house with relative humidity of 70% and 16 hrs/day photoperiod for the purpose of hardening. Confirmation of genetic stability of the *in vitro* raised banana plantlets of the present cultivar becomes imperative for the employment of the current method in future micropropagation programmes. For this purpose, molecular fingerprinting analysis using 11 ISSR primers was conducted with five randomly selected culture derived banana plants to compare with the mother plant.

Total genomic DNA was isolated from young tender leaves of mother plant growing in the garden as well as *in vitro* raised banana plant following the protocol of DNeasy® Plant Mini Kit-Qiagen (Part no.69104). The purity, size and integrity of DNA were determined by using a 0.8% agarose gel stained with ethidium bromide (EtBr) using gelpilot 1 kb plus ladder.

Amplification of DNA was carried out with 11 ISSR primers using a Veriti™96 well thermal cycler (Applied Biosystems by Thermo Fisher Scientific). PCR was performed with 25 µl reaction mixture containing genomic DNA (~30 ng/µl, measured by Nanodrop 2000c spectrophotometer), 10 mMdNTPs (Qiagen), 10X Taq buffer with 15 mM $MgCl_2$ (Qiagen), 10 µM each primer and 2.5 units/µl of Top Taq DNA polymerase (Qiagen). Amplification was carried out with initial temperature of 94°C for 5 min to denature the DNA followed by 44 cycles having three temperature ranges: 94°C for 1 min with varied temperatures as per melting temperatures of ISSR primers and 72°C for primer extension

followed by 72°C for 20 min. Amplification reaction was performed two times for each ISSR primer. Amplified DNA fragments were separated on 2% agarose gel electrophoresis stained with ethidium bromide (EtBr) and gels were visualized through gel documentation system (XR +, Bio-Rad).

The amplified DNA fragments generated by ISSR primers were scored with binary values '1' for presence and '0' for absence. Only clear and reproducible bands were scored. Binary data were used to calculate the level of monomorphism and polymorphism for each primer.

The data on different morphogenetic responses were expressed as mean ± Sd of five replicates per treatment and subjected to one-way ANOVA followed by DMRT at $p < 0.05$ for comparison to reveal any possible significant differences.

Results and Discussion

Simplification of culture needs by lengthening the period of culture incubation in MS supplemented with BAP, Kn, 2-iP in a particular concentration (4 mg/l) was carried out in the present study. Long term culture of *in vitro* derived shoot clusters subjected to the treatments revealed visible shoot multiplication differences and also a good response of root induction. Rate of shoot multiplication was better achieved in culture medium fortified with BAP (Fig. 1A) compared to other two treatments of Kn (Fig. 1B) and 2-iP (Fig. 1C). Maximum number of shoots was recorded in BAP supplemented media with an average shoot number of 8.00 ± 2.00 per cluster. This was followed by media containing Kn and 2-iP, respectively. However, effect of 2-iP treatment exhibited increased shoot growth (8.27 ± 0.89) cm in comparison to other two cytokinins (Table 1).

Relative superiority of BAP in shoot multiplication was also reported by many workers (Kim et al. 2003, Mishra and Chakraborty 2009, Mahmood and Hauser 2015). Potential of BAP in induction of shoot formation from meristematic explants in banana has been well documented in earlier works (Buah et al. 2010, Ferdous et al. 2015). Similar trend of high frequency *in vitro* shoot induction in BAP supplemented media followed by Kn was observed for banana cv. Grand Naine by Yadav et al. (2017). Observations on other relevant morphometric characters like mean number of leaves per shoot and mean leaf size revealed almost similar range of values. Although the treatment of Kn supplemented media yielded minimum mean number of leaves per shoot (3.37 ± 0.13) but has resulted in better growth of leaves in terms of size (3.48 ± 0.63 × 1.49 ± 0.31) cm^2 in comparison to other two treatments. Prolonged culture (8 weeks) in cytokinin enriched medium supported spontaneous root induction and growth without any supply of exogenous root inducing growth regulators which has been substantial with respect to simplification of culture needs. Root induction and production in all the treatments of cytokinin produced almost similar range of values for mean number of roots and root size (Table 1). Prolific rooting of the shooting clusters in almost all the treatments for long term culture incubation suggests facultative or non-obligatory use of root inducing

hormone for this particular banana cultivar. However, certain *in vitro*morphogenic response may result from endogenous accumulation of auxin or due to stress (Feher et al. 2001, Cueva Agila et al. 2013) and the same factors may be responsible for the development of roots in the present experiment. Relatively higher mean root size was recorded in media supplemented with Kn followed by 2-iP and BAP respectively.

Table 1. Morphometric characters of *Musav* sp. cultivar Gopi for 8 weeks in MS with BAP, Kn and 2-iP.

Morphometric characters (*Mean ± Sd)	Treatment with cytokinins (4 mg/l)		
	BAP	Kn	2-iP
No. of shoots	8.00 ± 2.00[a]	6.60 ± 2.41[a]	5.40 ± 1.34[a]
	(6.00 - 11.00)	(4.00 - 10.00)	(4.00 - 7.00)
No. of leaves	4.53 ± 0.65[b]	3.37 ± 0.13[c]	4.54 ± 0.92[b]
	(3.90 - 5.57)	(3.30 - 3.60)	(3.50 - 6.00)
No. of roots	4.43 ± 0.90[d]	4.76 ± 1.04[d]	4.57 ± 0.28[d]
	(3.33 - 5.71)	(3.40 - 6.00)	(4.33 - 5.00)
Size parameters (cm)			
Shoot size	6.51 ± 1.23[e]	7.58 ± 0.99[ef]	8.27±0.89[f]
	(5.01 - 7.88)	(6.46 - 8.75)	(7.33 - 9.37)
Leaf length	3.15 ± 0.71[g]	3.48 ± 0.63[g]3	0.32 ± 0.25[g]
	(1.99 - 3.89)	(2.70 - 4.15)	(3.07 - 3.67)
Leaf width	1.45 ± 0.27[h]	1.49 ± 0.31[h]	1.29 ± 0.15[h]
	(0.97 - 1.63)	(1.06 - 1.74)	(1.11 - 1.47)
Root size	5.38 ± 0.91[i]	6.09 ± 1.19[i]	5.85 ± 0.45[i]
	(3.87 - 6.21)	(4.86 - 7.50)	(5.33 - 6.59)

*Mean of five shoot clusters; Data represent mean ± Sd followed by different letters for significant difference within the rows using one way ANOVA and DMRT at $p < 0.05$; in parentheses range of minimum and maximum values.

In fact rooting at varying levels was found to occur in all the regenerated shoots without any exogenous input of auxin in long term culture. Development of root system ofthe elongated shoots was efficient and vigorous (Fig. 1F). In contrast, normal and rapid rooting of *in vitro* shoots was successfully achieved in media with IAA (2.00 mg/l) within a week of culture (Fig. 1E). Necessity of auxin application for better rooting of banana cultivars has been reported in previous works (Madhulata et al. 2006, Safarpour et al. 2017). Hardening of the *in vitro* regenerants in field conditions was successful with high survival rate and they were also healthy with no morphological abnormalities (Fig. 1G). Subsequent confirmation of genetic stability of the *in vitro* raised plantlets with the mother plant was carried out through ISSR markers which revealed true to type clonal nature. True to type plantlets with no genetic or morphological alteration is the outcome of a successful micropropagation protocol (Prakash et al. 2016, Safarpour et al. 2017).

Moreover, commercial banana production necessitates the testing of genetic stability of *in vitro* raised plantlets. Molecular fingerprinting analysis using 11 ISSR primers was done to check the genetic stability of five randomly selected culture derived banana plants and to compare them with the mother plant. Eleven primers revealed a total of 84 numbers of bands with an average 7.64 number bands per primer. The number of score

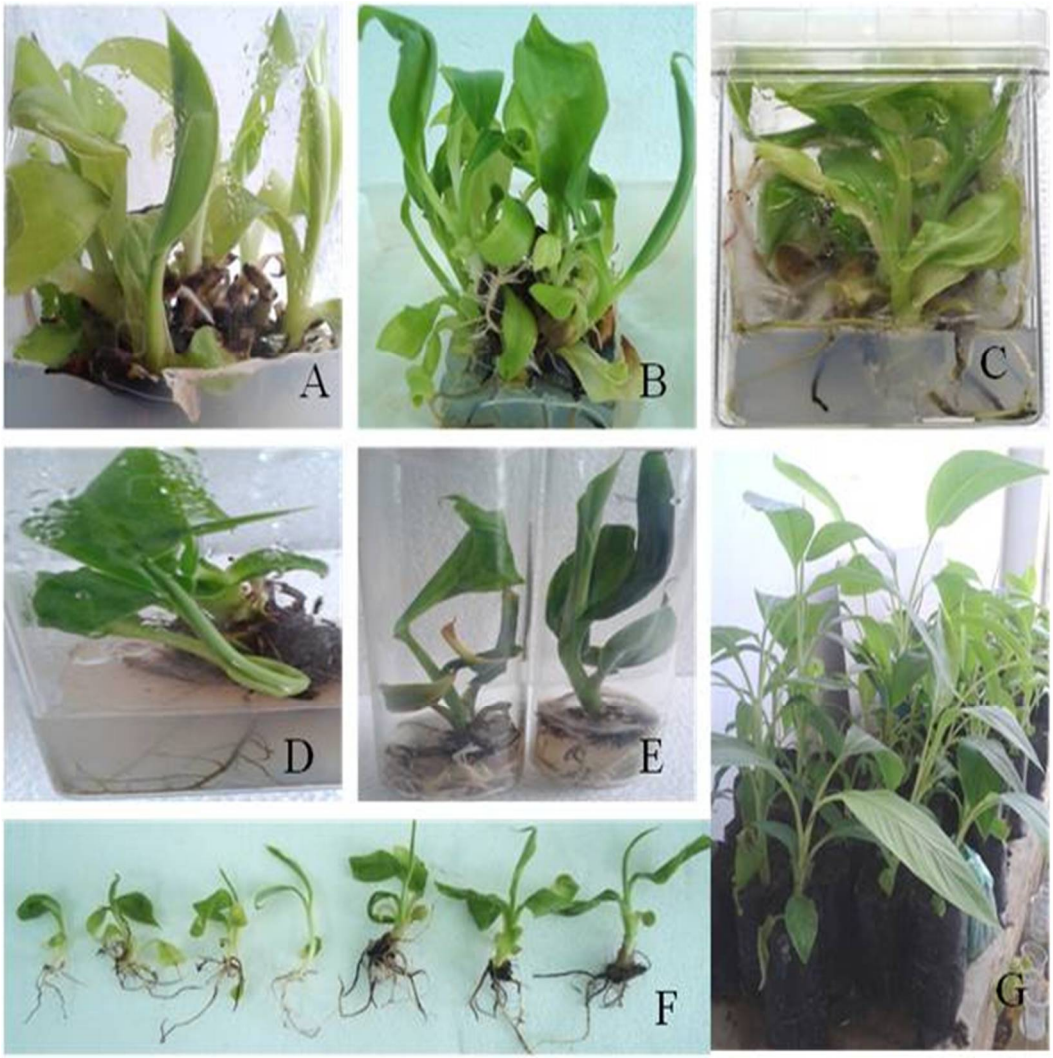

Fig. 1. Shooting clusters raised in prolonged culture in MS containing 4 mg/l (A) BAP, (B) 2iP and (C) Kn, (D) rooting of shoots in media containing low 2iP, (E) normal rooting of shoots in media containing IBA (2 mg/l), (F) rooted clones of the shoot cluster generated without any exogenous root inducing growth regulator and (G) successful hardening of the clones in potted soil.

bands per primer varied from 4 to 12 with 504 number of total amplified bands (Table 2). The maximum number of amplified band was recorded in UBC 808 with 72 numbers of bands (Fig. 2). Results of the present study indicated no detectable variation in the *in vitro* obtained clones of the banana cultivar Gopi through ISSR analysis. No polymorphism was detected among all the regenerated plants. Absence of genetic variability in tissue culture produced banana cultivar using ISSR markers has also been reported by Lakshmanan et al. (2007). However, some authors have reported genetic, epigenetic and phenotypic changes which occur during the *in vitro* regeneration process (Sharma et al. 2011, Singh et al. 2012). Somaclonal variation of culture derived banana cultivars was also reported by Nandhakumar et al. (2017).

Table 2. Banding pattern of ISSR primers among five randomly selected micropropagated and mother plants of *Musa* sp. cultivar Gopi.

ISSR primers	Primer sequence (5'-3')	NSB	MB	PB	PMB	Total number of amplified bands
UBC 808	$(AG)_8C$	8	8	0	100	48
UBC 815	$(CT)_8T$	5	5	0	100	30
UBC 824	$(TC)_8G$	8	8	0	100	48
UBC 840	$(GA)_8YT$	11	11	0	100	66
UBC 849	$(GT)_8CG$	6	6	0	100	36
UBC 855	$(AC)_8YT$	4	4	0	100	24
UBC 864	$(ATG)_6$	8	8	0	100	48
UBC 880	$(GGAGA)_3$	12	12	0	100	72
HB 15	$(GTG)_3GC$	9	9	0	100	54
ISSR 67	$(TC)_7CC$	4	4	0	100	24
ISSR Y11	$(GA)_8T$	9	9	0	100	54
Total		84	84	0	1100	504
Mean		7.64	7.64	0	100	45.82

NSB - Number of score band, PB - Polymorphic band, MB - Monomorphic band, PMB - Percentage of monomorphic band.

All the amplified bands of culture derived banana plants were homogenous and monomorphic in nature. Molecular fingerprinting study using ISSR markers of tissue culture derived plant revealed similar banding pattern with the mother plant. The result indicated that, ISSR primer could be useful for detecting genetic stability of *in vitro* raised banana plant. Therefore, clonal fidelity analysis of *in vitro* propagated banana plants revealed clonal in nature. Findings of the present study highlight on manifestation of long term incubation culture method for mass multiplication of *Musa* sp. cultivar Gopi through a different outlook of economic and labour feasibility. Reduction of certain obligatory requisites of culture method like subculture phases, exogenous supplementation of rooting hormone and omitting the rooting phase in root inducing

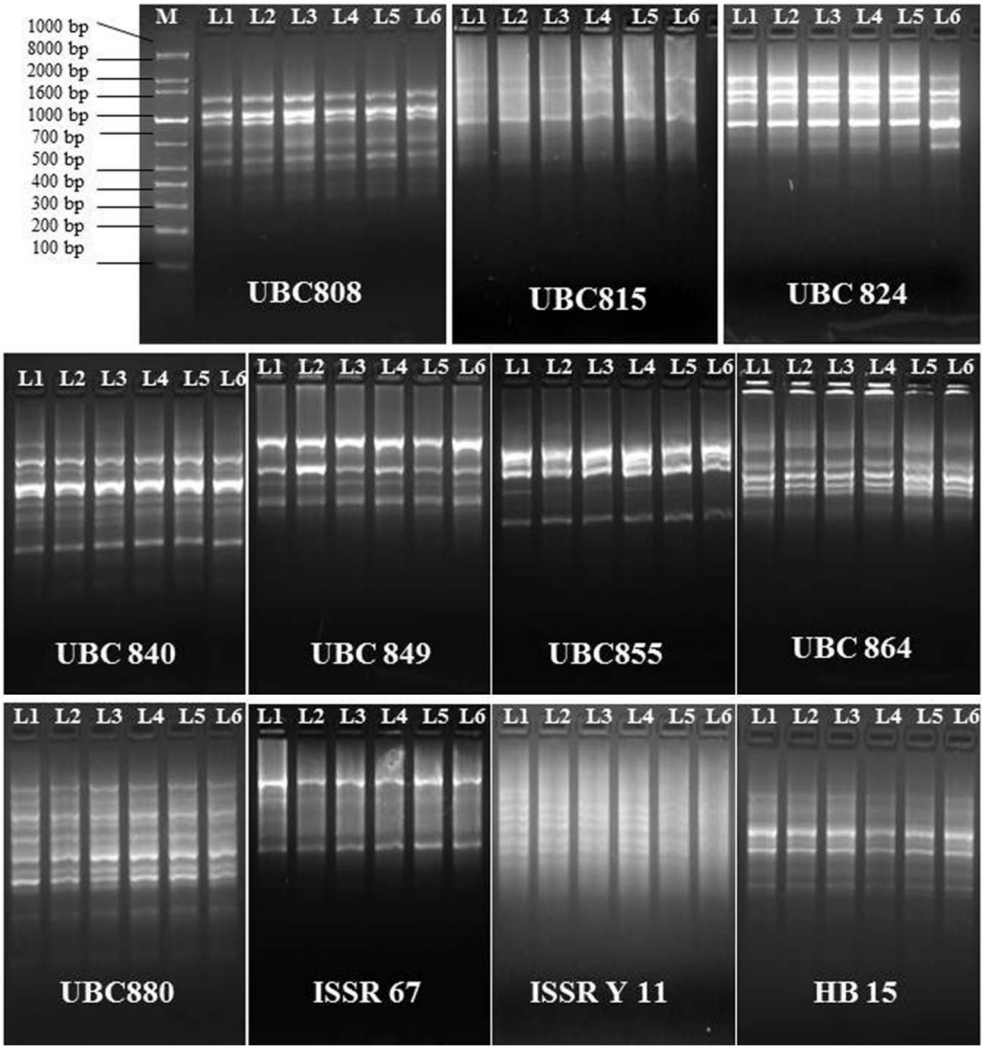

Fig. 2. ISSR profile of five randomly selected micropropagated and control plant of *Musa* sp. cultivar Gopi where, M represents 1 kb plus ladder, L1 - L5 represent five regenerated plants and L6 represents mother plant.

plant growth regulator along with high survival rate is profitable concerning the sophisticated requirements of tissue culture. In addition, establishment of genetic fidelity of the *in vitro* clones revealed by ISSR analysis suggests the suitability of the current method to produce true-to-type banana plantlets without any risk and consequent mass propagation of *Musa* sp. cultivar Gopi for preservation, food security and utilization in commercial industry.

Acknowledgements

Department of Biotechnology (DBT), Govt. of India provided financial support through a project (No. BCIL/NER-BPMC/2018/245).

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(Manuscript received on 17August, 2019; revised on 25 September, 2019)