In Vitro Propagation of an Edible Bamboo Bambusa Bambos and Assessment of Clonal Fidelity through Molecular Markers

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Abstract—**An efficient and reproducible protocol has been established through the technique of forced axillary branching for the propagation of an important edible bamboo species namely *Bambusa bambos*. High frequency multiple shoot induction was achieved from nodal segments collected from elite genotype on Murashige and Skoog's (MS) medium supplemented with 4.4 µM Benzylaminopurine (BAP) and 1.16 µM Kinetin (Kn). The size of explant and season greatly influenced the frequency of bud break. Rooting posed a major problem to be worked out in this particular species. Best rooting response was observed on 9.80 µM of Indole- 3 Butyric acid (IBA) with 60 ± 14.1 % rooting. *In vitro* raised plants were successfully acclimatized and established in the field conditions where they exhibited normal growth. In a bid to ascertain genetic fidelity, DNA was extracted by CTAB method and samples were analysed in 1.8% agarose gel electrophoresis. In the present study no variation was reported among the *in vitro* raised progeny and the mother plant in the banding profiles generated by the total of fifteen Random Amplified polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers. Hence, molecular analysis confirmed that these plants were genetically similar and can be used as elite plants.**

Index Terms—***Bambusa bambos*, *in vitro*, micropropagation, RAPD, ISSR**

I. Introduction

Bamboos are the most important forest species of Southeast Asian countries where they form the backbone of rural economy. Next to China, India is the second largest producer of bamboos in the world producing 4-6 million tones of bamboos annually, out of which 1.9 million tonnes are used in paper industry. Besides their application in paper industry, they are extensively used for house building, furniture making, floor tiles, for soil conservation, eating purposes and their leaves make an excellent cattle fodder. With renewed interest in bamboo propagation in India through Bamboo Mission Programme, the emphasis is on producing quality bamboo material on large scale and to introduce other economically important bamboo species. Among these, *Bambusa bambos* attains the greater significance. *Bambusa bambos* called as 'Giant Thorny' is a densely tufted bamboo growing up to a height of 25 m having multifarious uses [1]. Currently, International Union for Conservation of Nature (IUCN) Red list of endangered bamboo species has included *Bambusa bambos* as a priority species and hence needs conservation [2] Limitations in traditional propagation methods such as the use of offsets, branch cuttings together with unpredictable and long flowering cycle (40-60 years) warrants an urgent need for an alternative approach for developing efficient and reproducible protocol for its mass propagation. *In vitro* propagation or tissue culture technique holds tremendous potential for the production of high-quality planting material [3]. In this report we describe an efficient and reproducible micropropagation protocol for generating uniform clones through axillary branch proliferation using vegetative tissue derived from mature field-grown clumps of *B. bambos* and establishing the genetic fidelity through RAPD and ISSR markers. Earlier, [4] reported somatic embryogenesis in *B. bambos* which carries a higher risk of genetic instability due to somaclonal variations, thereby defeating the purpose of micropropagation. [5] reported multiple shoot proliferation from nodal explants but no rooting could be induced. None of these studies attempted to analyse the genetic fidelity of *in vitro* raised plants of *B. bambos*, which is of utmost importance in determining the practicality of any micropropagation protocol.

II. Material and Methods

The nodal explants were collected from the precocious branches of 5 years old healthy field grown plant of *B.bambos.* After washing with teepol solution and bavistin (0.1% w/v), explants were surface sterilized with

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0.1% (w/v) aqueous solution of $HgCl_2$ and were cultured on MS [6] medium augmented with different growth regulators, 2% sucrose and 0.8% agar or 0.2% phytagel with pH adjusted to 5.8 before the addition solidifying agent. All the inoculated cultures were incubated in growth room at a temperature of 25 ± 2ºC with a photoperiod of 16 hours per day with an illumination of 50 µmol/m^{-2}/s^{-1} at the level of cultures. Various factors like initial response of explant, contamination rate, bud break and survival percentage were considered for initiation of cultures. Basal MS medium supplemented with a combination of two cytokinins viz BAP (1.1 to 13.2 µM) and Kn (1.16 to 9.3 µM) with sucrose (1 to 3 %) was tested for standardization of axillary proliferation. For root induction, shoots were excised in clumps of 3-4 from multiple shoot bunches and transferred onto MS media in full, half and quarter strengths supplemented with IBA (4.90 to 24.5 µM) and NAA (5.37 to 24.16 µM) with 0.8% agar or 0.2% phytagel. The rooted plantlets were hardened on sand, soil and farmyard manure (1:1:1) under greenhouse conditions and finally transferred to full sunlight. To test the clonal fidelity, the DNA of the mother plant and *in vitro* raised hardened plants was extracted from young leaves by using modified Cetyl Trimethyl Ammonium Bromide (CTAB) method [7]. A total of Twenty five RAPD and ISSR primers at a concentration of 1 µM / reaction were scanned in the present study. PCR amplification was carried out in total volume of 25 µl containing 2 µl (20 to 25 ng) of genomic DNA. The reaction buffer for ISSR consisted of 2.5 µl Taq buffer, 1 µl $MgCl_2$, 0.15 µl dNTPs (10 mM each of dATP, dGTP, dTTP and dCTP), 1.5 µl primer, 0.17 µl Taq polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 17.68 µl water. PCR amplification was performed in a DNA thermal cycler which was programmed for initial DNA denaturation at 94°C for 4 min, followed by 44 cycles of 1 minute denaturation at 94°C, 1 minute annealing (temperature specific to the primer) and 1 minute extension at 72°C, with a final extension at 72°C for 7 minutes. For RAPD, reaction buffer consisted of 2.5 µl Taq buffer, 0.5 µl $MgCl_2$, 0.2 µl dNTPs, 1.5 µl primer, 0.17 µl Taq polymerase and 18.13 µl water. PCR amplification consisted of initial denaturation at 94°C for 5 minutes, followed by 45 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 37°C and 2 minutes extension at 72°C, with a final extension at 72°C for 7 minutes. The amplified products were resolved by electrophoresis on 1.8% agarose gel in tris-borate EDTA (TBE) buffer stained with ethidium bromide. The fragment sizes were estimated with 100 and 500 bp DNA ladders (Bangalore Genei Pvt. Ltd, Bangalore, India).

III. Results and Discussion

A. Initiation of Aseptic Cultures and Factors Affecting Percentage Bud Break

Due to the considerable variations in the environmental conditions during different periods of the year, maturity status of the explant varied with season, hence response of explant to culture initiation also varied. The best period for raising aseptic cultures was spring (February and March) when 93% bud break was observed. Rainy season had almost an equal frequency of bud break but the rate of contamination was very high. Summers and winters were the least preferred seasons for the initiation of cultures as percentage of bud break was very low being 37 and 50% respectively. The dependence of bud break on external factors like the condition and the health of the mother plant has been reported earlier by [8], [9] and [10]. Size of explant also influenced the time taken for the bud break. Small sized explants (5-7 mm) did not respond well in the culture whereas explants of 25 mm took least number of days for initiation of sprouting probably due to greater concentration of endogenous hormones supporting bud break.

B. Shoot Multiplication

For inducing sprouting, nodal explants were inoculated on MS medium with or without cytokinins. Nodal explants cultured on MS basal medium without any cytokinin took more time to sprout (25 days) where as the nodal explants sprouted within 15 days of inoculation on MS medium supplemented cytokinin (Fig. 1a). The sprouted buds were excised from the nodal segment and transferred onto MS medium containing different concentrations and combinations of cytokinins like BAP (1.1 to 13.2 µM) and Kn (1.16 to 9.3 µM). Axillary shoot proliferation occurred on MS medium supplemented with different concentrations of BAP but 4.4 µM of BAP gave maximum multiplication rate. A significant increase in the shoot number was observed when BAP (4.4 µM) was used in conjunction with Kn (1.16 µM) forming 6-8 shoots after 3 weeks (Fig. 1b). The shoots multiplied further forming 21.70^{a} ± 2.40 shoots after 6 weeks (Fig. 1c).

C. Rooting and Acclimatization

In general, rooting is a main bottleneck while carrying out *in vitro* multiplication of bamboos and has been a major problem to be worked out in this particular species. It was observed that rooting was more effectively induced when clusters of shoots rather than individual shoot were used. The clumps of 3 to 4 shoots were inoculated on MS medium supplemented with different concentrations and combinations of auxins. Out of all the auxins tried, initiation and growth of roots was observed on IBA (9.80 µM) supplemented medium where rooting occurred in 60% of cultures (Fig. 1d & e). The role of IBA and NAA in root induction had been reported earlier in other bamboos like *Bambusa vulgaris* by [11]; in *B. oldhamii* by [12] and *Dendrocalamus asper* by [13]. Complete plantlets were hardened on soil: sand: farmyard manure (1:1:1) by gradually lowering the relative humidity and were successfully transferred from *in vitro* to natural environmental conditions with 80% survival rate. The plants showed well developed root and shoot systems and all the plants are thriving very well in field conditions with no apparent phenotypic variations (Fig. 1f).

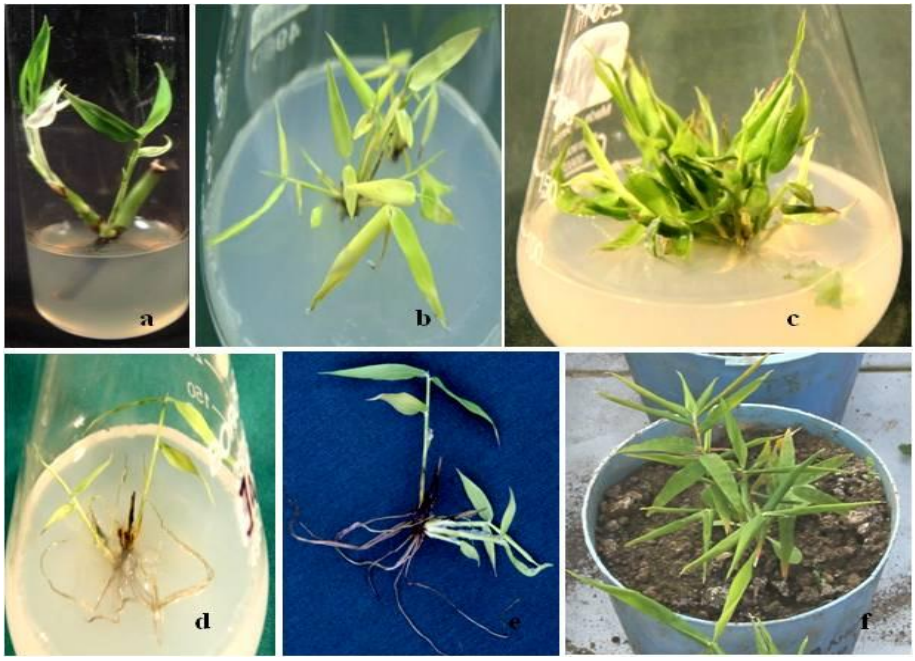

Figure 1. a. Bud break on MS+ BAP (4.4 µM) b. Initiation of multiple shoots on MS + 4.4 µM BAP and 1.16 µM Kn after 3 weeks. c. Proliferation of numerous shoots after 6 weeks. d. Root induction on 9.80 µM IBA. e. A complete plantlet formed f. 2- months-old hardened plant in field condition

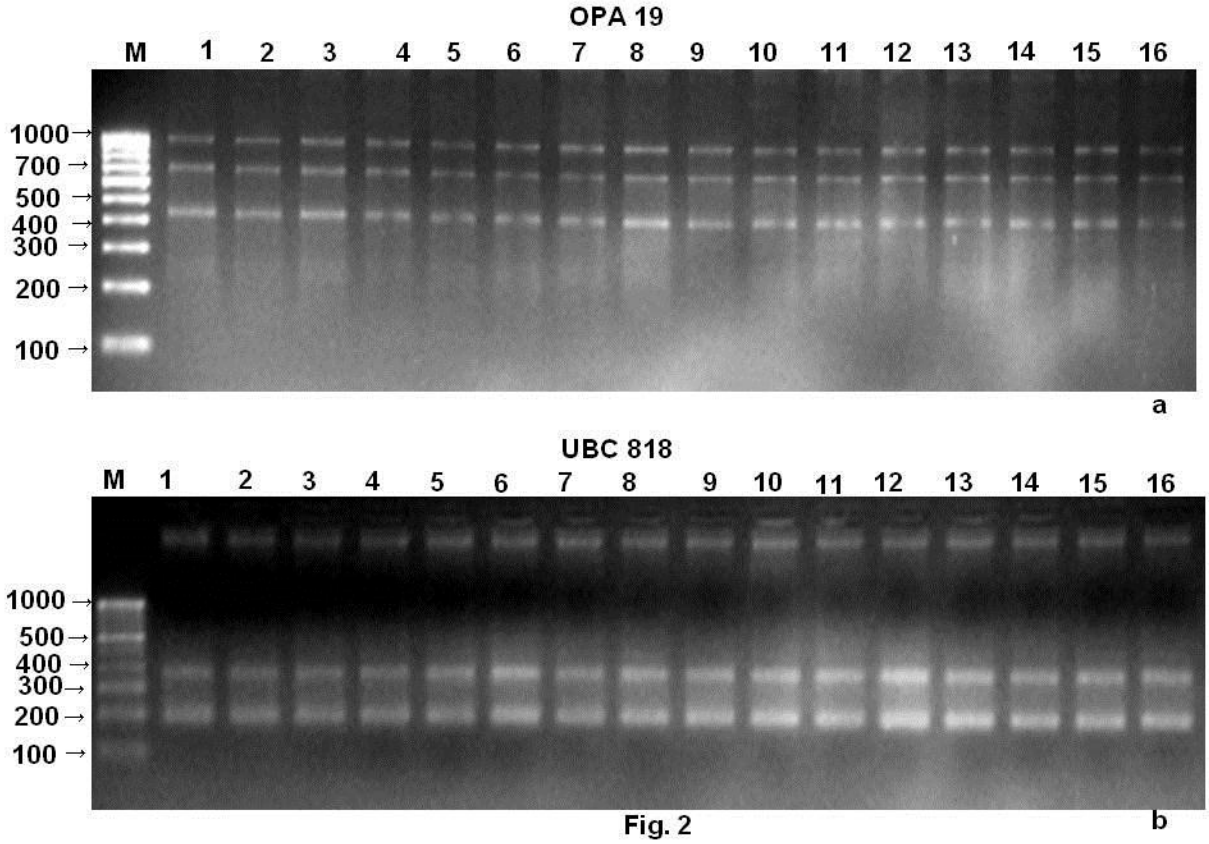

Figure 2. Amplifications produced by using RAPD marker OPA 19. Amplifications produced by using ISSR marker UBC 818. Lane M represent ladder, lane 1 is the mother plant and lane 2-16 represent the tissue culture (TC) – raised plants.

D. Clonal Fidelity Using RAPD and ISSR as Molecular Markers

The scarcity of reports on ascertaining the genetic fidelity of tissue culture raised plantlets can jeopardise the quality of micropropogated plants, especially in perennials like bamboos where any undesirable variant would last for several years [14]. Therefore, it is pertinent to screen the regenerants for the occurrence of any

somaclonal variations. In the present investigation, for ascertaining the clonal fidelity, 15 randomly selected plants were subjected to RAPD and ISSR analyses. Out of 15 RAPD markers scanned only 10 primers produced amplicons as shown in Table I. In case of ISSR analyses using 10 markers only, 5 markers gave scorable bands as depicted in Table II. Out of total 25 scanned markers, amplified products in the range of 100 to 1500 bp were obtained as shown in Fig. 2. Optimum T_m for RAPD markers falls near 37º C and that for ISSR markers the range falls between 45.4 to 54.3 ºC. For RAPD analysis, OP series gave the best amplification. For ISSR analysis, UBC 818 gave the maximum amplified products in the range of 200 to 1400 bp. We found that all banding profiles from the micropropagated plants were monomorphic and similar to those of mother plant. Hence, clonal fidelity was established with no apparent genetic and epigenetic variations. Earlier, [9] established the clonal fidelity of regenerants of *Bambusa tulda* and *B. balcooa* using only four markers to assess the genetic uniformity among the regenerants. [15] assessed the clonal fidelity of *in vitro* raised plants of *Dendrocalamus hamiltonii* using 18 RAPD markers. Later, [16] employed 30 RAPD and 27 ISSR markers to validate the clonal fidelity of *in vitro* raised *Guadua angustifolia* plantlets through the axillary bud proliferation. However, there is no report available on the comparative genetic stability of regenerants and mother plant of *Bambusa bambos* using RAPD and ISSR markers. In the present study, we did not find any polymorphism during the RAPD and ISSR analysis of *in vitro* raised clones, thus confirming true to type nature of *in vitro* raised plants.

TABLE I. RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS UTILIZED TO VERIFY BAMBUSA BAMBOS CLONES

Primers	5'-3' motif	Scorable bands	Monomorphic bands	Polymorphic bands	Range of Amplification
OPA 01	CAG GCC CTT C	3	3	0	1000, 1100, 1300
OPA 02	TGC CGA GCT C	3	3	0	400, 900, 1000
OPA 19	CAA ACG TCG G	3	3	0	450, 700, 1000
OPT 10	CCT TCG GAA G	3	3	0	300, 500, 1500
OPT 18	GAT GCC AGA C	2	2	0	800, 900
OPO 06	CCA CGG GAA G	1	1	0	1500
OPO 07	CAG CAC TGA C	2	2	0	450, 1400
OPO 08	CCT CCA GTG T	2	2	0	200, 1300
OPO 15	TGG CGT CCT T	2	2	0	800, 1500
OPO 18	CTC GCT ATC C	2	2	0	200, 1300
Total	10	23	23	0	200-1500

OP Series sequences of Operon Technologies – Alameda, USA

TABLE II: THE INTER SIMPLE SEQUENCE REPEATS (ISSR) MARKERS UTILIZED TO VERIFY BAMBUSA BAMBOS CLONES

Primers	5'-3' motif	T_m ºC	T_m ºC	Scorable Bands	Monomorphic Bands	Polymorphic bands	Range of Amplification (bp)
UBC 810	$(GA)_9$ T	45.4	42.5	4	4	0	100, 400, 450, 1000
UBC 812	$(GA)_8$ A	45.7	41.5	2	2	0	100, 350
UBC 818	$(CAC\ ACA)_2$ CAC AG	51.0	48.5	3	3	0	200, 400, 1400
UBC 834	$(GA)_9$ T	49.2	46.0	3	3	0	350, 400, 700
UBC 857	$(GA)_9$ T	54.3	50.5	2	2	0	400, 1500
Total	5	-	-	14	14	0	100-1500

UBC Series sequences of University of British Columbia, Canada; Y = (C,T)

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