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Ascertaining clonal fidelity of tissue culture raised plants of *Bambusa balcooa* Roxb. using inter simple sequence repeat markers

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Abstract One of the major applications of plant tissue culture technology is that the regenerants are genetically identical to the mother plant. Although, of the various methods of in vitro propagation, axillary branching method is regarded as the safest, the possibility of genetic variation (somaclonal variation) cannot be ruled out even in this method. In the present study, inter simple sequence repeat (ISSR) marker assay was employed to validate the clonal fidelity of in vitro raised *Bambusa balcooa* plantlets multiplied by enhanced axillary proliferation up to 33 passages. Fifteen ISSR primers generated a total of 99 amplicons among the tissue-cultured progenies. Analysis of ISSR patterns revealed that the bands were shared by both the parent clump and the in vitro raised plants confirming the genetic stability in the latter.

Keywords *Bambusa balcooa* · Clonal fidelity · Somaclonal variation · ISSR · Axillary branching

Introduction

Bambusa balcooa Roxb., a member of the family Poaceae, is a multi-purpose bamboo species native to the Indian subcontinent. The species is widely distributed in different parts of India up to an altitude of 600 m. Highly valued for its strong culms, *B. balcooa* is regarded as one of the most suitable species for building purposes and scaffolding (Tewari 1992). Young shoots are edible and bitter in taste. Phytosterols, present in the fermented succulent shoots of *B. balcooa* are the precursors of many pharmaceutically active steroids (Sarangthem and Singh 2003).

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Bambusa balcooa has a long flowering cycle of 55–60 years (Tewari 1992). The clump dies after flowering without setting any seed. As a result, it can be propagated only through asexual means. But vegetative propagation through culm cuttings, branch cuttings, rhizomes is difficult on account of fewer and bulky propagules and season specificity. In vitro techniques of propagation can be of immense value in mass propagation of this species and thereby meeting its demand. Of the various methods of in vitro propagation, axillary branching is least susceptible to genetic modifications that might occur under in vitro conditions (Shenoy and Vasil 1992). However, even in axillary branching method there is every possibility of formation of somaclonal variants, thereby hindering the commercialisation of any micropropagation protocol, as reported in *Hagenia abyssinica* (Feyissa et al. 2007), apple rootstock MM106 (Modgil et al. 2005), *Robinia pseudoacacia* (Bindiya and Kanwar 2003) and *Populus deltoides* (Rani et al. 1995).

There are various methods known which can be used to detect tissue culture induced variations in plants. These include morphological, cytological, protein and molecular markers. Out of these, molecular marker techniques are more rapid, sensitive and reliable with regard to detection of somaclonal variation in comparison to phenotypic and karyologic analyses. Efficiency of a molecular marker technique lies in the extent of polymorphism that it can detect in the species under investigation.

Microsatellites or simple sequence repeats (SSRs) consist of short tandem repeats based on 2–5 bp motifs and are useful as genetic markers for comparative analysis and mapping of genomes (Weber and May 1989). The detected polymorphism is based on the number of di-, tri- or tetra-nucleotide repeats. However, construction of genomic library, synthesis of specific primers from flanking sequences is time-consuming and expensive. In addition, flanking regions must be known to generate polymerase chain reaction (PCR) primers (Leroy et al. 2001). ISSR, a variant of the SSRs technique described by Zietkiewicz et al. (1994) uses microsatellite oligonucleotides as primers that amplify genomic segments different from the repeat region itself. This approach employs oligonucleotides (14–22 bp) based on a simple sequence repeat anchored or not at their 5′- or 3′-end by two to four arbitrarily chosen nucleotides. This triggers site-specific annealing and initiates PCR amplification of genomic segments which are flanked by inversely oriented and closely spaced repeat sequences (Leroy et al. 2000). Such amplification does not require prior information on the genome sequence and leads to multilocus and highly polymorphic patterns (Tsumara et al. 1996; Nagaoke and Ogihara 1997). ISSR markers are universal, quick, easy to apply, and they have the reproducibility of SSR markers because of the longer length of their primers (Bornet and Branchard 2001). Detection of genetic uniformity through the use of ISSR marker assay has been successfully demonstrated in micropropagated plants of *Crataeva magna* (Bopana and Saxena 2009), *Swertia chirayita* (Joshi and Dhawan 2007), *Musa* spp. (Ray et al. 2006) and almond (Martins et al. 2004).

ISSRs segregate mostly as dominant markers (Gupta et al. 1994; Tsumara et al. 1996). However, they have also been shown to segregate as co-dominant markers thus enabling distinction between homozygotes and heterozygotes (Wang et al. 1998; Sankar and Moore 2001). Further, there is the possibility, as in RAPD, that fragments with the same mobility originate from non-homologous regions, which can contribute to some distortion in the estimates of genetic similarities (Sanchez de la Hoz et al. 1996).

The present communication describes the use of ISSR technique in ascertaining the clonal fidelity of tissue-culture raised plantlets of *B. balcooa* multiplied up to the 33rd passage. A single clone of *B. balcooa*, growing at Gualpahari, Haryana, India, was used to produce and dispatch nearly 22,000 tissue cultured plants by axillary branching method.

Materials and methods

Plant material

Single node segments measuring 2.5–3.0 cm in length, with unsprouted bud, were excised from lateral branches of a well-established plant of *B. balcooa*. The nodal segments were surface sterilized and inoculated in Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with different cytokinins, 3% sucrose and 0.2% gelrite. The in vitro formed shoots were multiplied in 350 ml jars containing 30 ml liquid medium. At the end of every 3-weeks passage, the proliferating shoot clusters were divided into 5–8 shoot clusters. MS medium supplemented with various growth regulators resulted in multiplication of shoots at a consistent rate. High rooting frequency was achieved when clusters of 5–6 shoots were rooted on half strength MS medium (major salts reduced to half) supplemented with various auxins. Plantlets derived were successfully hardened following sequential acclimatization of plants to greenhouse, polyhouse and shade net. All the experiments were repeated thrice with at least 24 plantlets per replicate.

Source of leaf material

The leaves of the mother plant of *B. balcooa*, in vitro grown shoots in 16th, 20th, 26th and 33rd passages and the hardened plant growing in the field were used for ISSR analysis. The leaf samples were lyophilized in the 'Virtis freezemobile G' lyophiliser for 48 h at −70°C. *Bambusa nutans* was taken as an outlier to check the suitability of the primers for their ability to detect polymorphism in the species under consideration.

Genomic DNA isolation and DNA quantification

Total DNA was extracted following a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method described by Doyle and Doyle (1990). Purified total DNA was quantified by electrophoresis in 0.8% agarose gel (Sigma, Ultra-pure) in 0.5× Tris Borate EDTA (TBE) buffer at 30 mA (60 V) for 2–3 h to allow proper resolution. Uncut lambda DNA (25 ng/µl) was used as a standard molecular weight marker to quantify genomic DNA. The gel was stained by ethidium bromide (Sigma) with final concentration of 0.5 µg/ml, visualized under UV and photographed using a gel documentation system AlphaImager® EC (Alpha Innotech Corporation, ChemiImager™ Ready, CA, USA).

PCR amplification conditions

Twenty-three University of British Columbia (UBC, Vancouver, Canada) ISSR primers were used in the study to ascertain the reproducibility and clarity of bands. PCR was performed in a volume of 20 µl PCR reaction containing 10× PCR buffer (Biotools, SA), 50 mM $MgCl_2$ (Biotools, SA), 10 mM dNTPs (Promega, WI, USA), 1 unit of *Taq* DNA polymerase (Biotools, SA), 10 mM primer (UBC) and 25 ng of genomic DNA. DNA amplification was performed in a 'Bio-Rad DNA Engine' (Peltier Thermal Cycler) programmed for an initial denaturation step at 94°C for 2 min and 35 cycles as per the details below:

- 30 s at 94°C (Denaturation)
- 30 s at 42°C (Annealing)
- 1 min at 72°C (Extension); and one final extension of

- 5 min at 72°C (Extension)
- 4°C (Soak temperature)

Amplified DNA fragments were separated on a 2% agarose gel (United States Biochemicals, Cleveland, OH, USA) using 0.5× Tris–borate EDTA buffer and stained with ethidium bromide. The size of the amplification products was estimated from a 100 bp DNA ladder (New England Biolabs Inc.).

Electrophoresis was carried out at 80 mV for 3–4 h and the gels were visualised and photographed using a gel documentation system AlphaImager® EC (Alpha Innotech Corporation, ChemiImager™ Ready, CA, USA).

Data analysis

Only consistently reproducible, well-resolved fragments, ranging from 100 to 1,600 bp were scored as present or absent for ISSR markers in each sample.

Results and discussion

In vitro propagation through axillary branching has been used in the micropropagation of several bamboos such as *Guadua angustifolia* (Jimenez et al. 2007), *Dendrocalamus asper* (Arya et al. 1999), *Dendrocalamus longispathus* (Saxena and Bhojwani 1993) and *Bambusa tulda* (Saxena 1990). Although, all the tissue cultured plants are expected to be genetically identical yet the possibility of some genetic variation emerging during the in vitro process cannot be ruled out. Tissue culture induced phenotypic and genotypic variations are collectively termed as 'Somaclonal variation' (Larkin and Scowcroft 1981). They mostly occur as a result of the stress induced by the tissue culture process causing alteration in DNA methylation patterns, DNA damage and mutation, alteration of cell's ability to repair damaged and mutated DNA (Leroy et al. 2000), chromosomal rearrangements and point mutations (Kaeppler and Phillips 1993).

Though there are several reports on tissue culture of bamboos but only a single report confirming the clonal fidelity of bamboo plantlets raised through axillary branching method. The lack of reports on ascertaining the genetic fidelity of tissue culture raised plantlets could lead to serious consequences, especially in perennials like bamboo where any undesirable variant would last for several years. Therefore, it is advisable to screen for the occurrence of somaclonal variation (if any) among the regenerants at regular intervals. The present study was conducted to screen the tissue culture raised plantlets of *B. balcooa* for somaclonal variation by employing ISSR marker assay. In the present study, the primers suitable for ISSR studies in *B. balcooa* were identified and the PCR conditions have been optimized. Twenty-three ISSR primers were used initially for screening because not all the randomly selected primers would produce amplification. This information would be useful when tissue culture studies are extended to other clones.

Out of a total of 23 primers screened, only 15 primers produced amplification. The assay with 15 ISSR primers generated 99 scorable band classes, ranging from 100 to 1,600 bp. The number of bands for each primer varied from 3 to 10, with an average of 6.6 bands per ISSR primer. A total of 1,683 bands (no. of plantlets analysed × no. of band classes with all ISSR primers) were generated which were monomorphic across all the *B. balcooa* plantlets analysed. No polymorphic bands were observed in parent plant and the tissue-culture raised progeny whereas a polymorphic banding pattern was obtained with *B. nutans*

Table 1 The PCR amplicons obtained with ISSR primers used to ascertain the clonal fidelity in micropropagated plants of *B. balcooa*

ISSR primer	Primer sequence (5′–3′)	G + C content (%)	Tm (°C)	Monomorphic bands in TC-raised plant + outlier	No. of bands amplified only in the outlier (*B. nutans*)	Total number of bands amplified	Size range (bp)
UBC 801	$(AT)_8$ T	0	25	No amplification	No amplification	No amplification	–
UBC 802	$(AT)_8$ G	6	28	No amplification	No amplification	No amplification	–
UBC 805	$(TA)_8$ C	6	28	No amplification	No amplification	No amplification	–
UBC 806	$(TA)_8$ G	6	28	No amplification	No amplification	No amplification	–
UBC 812	$(GA)_8$ A	47	45	7	4	11	300–1,200
UBC 813	$(CT)_8$ T	47	45	5	1	6	700–1,200
UBC 814	$(CT)_8$ A	47	45	7	0	7	500–1,200
UBC 815	$(CA)_8$ G	53	47	No amplification	No amplification	No amplification	–
UBC 818	$(CA)_8$ G	53	47	6	5	11	500–1,200
UBC 819	$(GT)_8$ A	47	45	No amplification	No amplification	No amplification	–
UBC 830	$(TG)_8$ G	53	47	6	1	7	500–1,000
UBC 836	$(AG)_8$ YA	47	45	10	6	16	350–1,000
UBC 838	$(TA)_8$ RC	6	28	No amplification	No amplification	No amplification	–
UBC 840	$(GA)_8$ YT	47	45	9	7	16	500–1,400
UBC 841	$(GA)_8$ YC	53	47	7	6	13	300–700
UBC 842	$(GA)_8$ YG	53	47	7	4	11	100–700
UBC 843	$(CT)_8$ RG	53	47	7	5	12	400–1,600
UBC 848	$(CA)_8$ RG	53	47	7	5	12	300–1,200
UBC 850	$(GT)_8$ YC	53	47	8	7	15	300–1,000
UBC 852	$(TC)_8$ RA	47	45	3	3	6	500–1,000
UBC 857	$(AC)_8$ YG	53	47	5	2	7	500–900
UBC 860	$(TG)_8$ RA	47	45	5	0	5	500–1,200
UBC 868	$(GAA)_6$	33	41	No amplification	No amplification	No amplification	–
Total number of bands produced				99	56	155	

Where Y = C, T; R = A, G

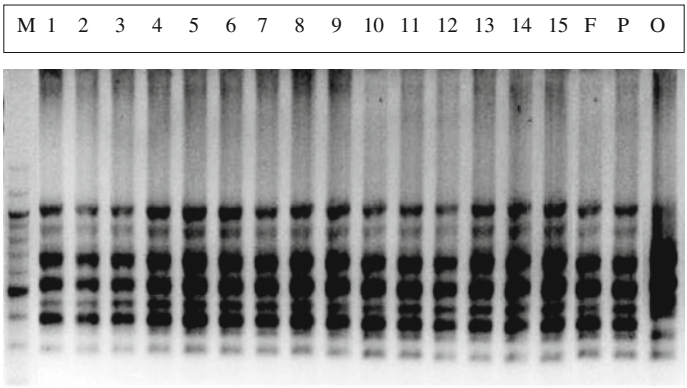

Fig. 1 Amplification produced by UBC primer 850. *Lane M* represents 100 bp ladder, *Lanes 1–4* represent the tissue-culture raised progenies in 16th passage, *Lanes 5–8* represent the progenies in 20th passage, *Lanes 9–12* represent the progenies in 26th passage and *Lanes 13–15* represent those in 33rd passage. *Lane F* represents the in vitro raised plant transplanted to the field. *Lane P* represents the parent plant of *B. balcooa*. *Lane 0* represents the outlier, *B. nutans*

acting as an outlier (Table 1; Fig. 1). Further, the polymorphic bands scored for the outlier proved that the primers employed were competent enough to distinguish the plantlets based on genetic variations. The uniform banding profile among the tissue culture raised progeny of *B. balcooa* confirmed genetic fidelity.

The correlation between the time of in vitro culture and the extent of genetic instability has been cited in literature (Tremblay et al. 1999; Yang et al. 1999; Lorz and Scowcroft 1983). However, contrary to this hypothesis, the length of the culture period (33 passages or more than 2 years) with regular subculturing did not affect the genetic integrity of the progeny of *B. balcooa*. The band profiles were monomorphic across all the samples analysed up to 33 passages. Similar results were also reported by Panda et al. (2007) in *Curcuma longa*, Joshi and Dhawan (2007) in *Swertia chirayita* and Martins et al. (2004) in almond plantlets where the cultures were maintained for a period of 26 months, 44 months and 4 years, respectively. The retention of clonal uniformity for prolonged period under in vitro conditions has immense commercial significance. It has been reported that initiation is difficult in *B. balcooa* owing to season specificity, persistent contamination, phenolic exudation, etc. (Das and Pal 2005b). Thus, conducting initiation after every 12–15 passages is not commercially viable. Therefore, if the tissue cultured plants retain genetic uniformity for prolonged culture duration, continuous shoot multiplication could be attained. This is an important finding in reducing the overall cost of production.

In an earlier report, Das and Pal (2005a) established the clonal fidelity of regenerants of *B. tulda* and *B. balcooa* by random amplified polymorphic DNA (RAPD) analysis. They used only four RAPD primers to assess the genetic uniformity among the regenerants of both the species. As compared to RAPD, ISSR marker assay reveals larger number of polymorphic fragments per primer than RAPD because of the occurrence of abundant SSR regions. Longer primers of ISSR (14–22 bp), than RAPD primers (10 bp), have higher annealing temperatures. Higher annealing temperatures mean more stringent primer annealing conditions, which lead to greater consistency; however, a low annealing temperature may increase non-specific amplification, leading to artefact bands (Bornet and Branchard 2001). Since, simple sequence based repeat primers target the fast evolving hypervariable sequences (Tautz 1989), ISSR markers are considered suitable to detect

variations or ascertain clonal fidelity among tissue culture produced plants. For instance, Ray et al. (2006) concluded that ISSR detected more polymorphism than RAPD in two *Musa* spp. cultivars Robusta and Giant Governor. The percentage of polymorphic loci by RAPD and ISSR were found to be 1.75, 5.08 in Robusta and 0.83, 5.0 in Giant Governor, respectively.

In our study, no variability was detected among the TC-plantlets by ISSR marker assay; we can therefore say that the tissue culture raised plantlets developed in the existing study did not reveal any somaclonal variation throughout the 33 passages. Thus, ISSR markers are highly efficient to ascertain the clonal fidelity of tissue culture raised progenies of *B. balcooa.* The in vitro clonal propagation using preformed organs such as axillary buds circumvents dedifferentiation or redifferentiation of cells or tissues, avoiding genomic aberrations and consequently maintaining the clonal fidelity of in vitro raised plantlets of *B. balcooa*.

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