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SHORT COMMUNICATION

Genetic homogeneity revealed in micropropagated *Bauhinia racemosa* Lam. using gene targeted markers CBDP and SCoT

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Abstract Two gene targeted markers i.e. CAAT box-derived polymorphism (CBDP) and start codon targeted (SCoT) polymorphism were applied to analyze the genetic stability of in vitro propagated plants of *Bauhinia racemosa* Lam. multiplied by enhanced axillary shoot proliferation of mature tree derived nodal explant. Nine randomly selected micropropagated plants of 1 year age were subjected to molecular analysis. The isolated genomic DNA samples were subjected to PCR amplification with a total of 61 primers (25 CBDP and 36 SCoT) out of which 39 primers (21 CBDP and 18 SCoT) produced scorable amplicons. A total of 97 and 88 clear, distinct and reproducible amplicons were produced by CBDP and SCoT primers, respectively. The monomorphic banding pattern obtained through all the tested primers corroborated the true to type nature of in vitro propagated plants of *B. racemosa*.

Keywords Gene targeted · Genetic stability · Molecular markers · Plant regeneration · Somaclonal variation

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Introduction

Significance of tissue culture-based propagation techniques reside in the fact that regenerated plant population should be genetically and morphologically uniform to their parent line (Sarmast et al. 2012). The in vitro development of plants is governed by various factors i.e. different types, age and genetic makeup of explant, method employed for regeneration, given nutrients and PGRs, controlled micro-climatic conditions (light, temperature, and relative humidity), ploidy levels and maintenance as well as age of in vitro cultures (Rani and Raina 2000; Bhojwani and Dantu 2013). These factors are supposed to destabilize the genetic structure due to phenotypic mutations, cytological abnormalities, changes in sequences and retrotransposon activation (Larkin and Scowcroft 1981; Kaeppler et al. 2000; Saker et al. 2000; Renau-Morata et al. 2005; Saha et al. 2016). They also cause the epigenetic modifications through changing the level and distribution of DNA methylation, histone modifications (methylation and acetylation) and microRNA induced variations in micropropagated plants which results into somaclonal variations (Us-camas et al. 2014). These genetic variabilites are considered as a major drawback of micropropagation system (Rout et al. 2006; Brito et al. 2010). However, various studies suggest that in vitro cultures established from stimulation of axillary/apical buds generate plantlets that are considered to be the most reliable for clonal multiplication due to maintenance of genetic integrity and least susceptibility of these explants to genetic variations (Negi and Saxena 2010; Singh et al. 2012; Rathore et al. 2014). However, possibility of somaclonal variations could not be overlooked completely (Nookaraju and Agrawal 2012) as they cause off-types which can seriously limit the commercial value of micropropagated plants (Oh et al. 2007).

Thus, in order to overcome the possibility of deleterious effects on plant growth and severe economic impact caused by somaclonal variations, genetic stability of micropropagated plants should be validated at early stage of plant growth especially in woody trees (Chuang et al. 2009; Marum et al. 2009; Kilinç et al. 2015).

Traditionally, genetic stability of micropropagated plants has been evaluated using cytological, phenotypic and protein-based methods. In recent years however, DNA-based molecular markers have achieved paramount success in validating the genetic stability of in vitro raised plants and bypassed the dependence on morphological and phytochemical diagnosis (Chavan et al. 2015). These molecular marker techniques generate reliable and reproducible results without being influenced by environmental factors on age, tissue and developmental stage of the plant. In most cases, arbitrarily amplified markers like RAPD (random amplified polymorphic DNA) and ISSR (inter-simple sequence repeat) or co-dominant SSR (simple sequence repeat) have been successfully employed for validation of genetic homogeneity of micropropagated trees (Rani et al. 1995; Martins et al. 2004; Asthana et al. 2011; Rai et al. 2012). Recently, gene targeted markers such as CBDP and SCoT have gained considerable importance in molecular analysis of plants. CBDP, a promoter-targeted marker uses the nucleotide sequence of CAAT box in plant genes (Singh et al. 2014). CAAT box, a regulatory element has a distinct pattern of nucleotides with a consensus sequence GGCCAATCT located ~ 80 bp upstream of the start codon in eukaryote genes and plays an important role during transcription (Benoist et al. 1980). Another gene targeted marker, SCoT exploits the gene regions flanking the ATG initiation codon (Collard and Mackill 2009). Some of the common features of both markers are, more reproducibility than arbitrary markers i.e. RAPD; use of single primer (reverse and forward) of 18 nucleotides; no prior sequence requirement; visualization on agarose gel electrophoresis. While testing genetic homogeneity, it has been recommended to use two or more primer types in order to target a wider part of the genome under study (Martins et al. 2004).

Bauhinia racemosa Lam. is a leguminous tree of family Caesalpiniaceae and is commonly known as apta tree or sonpatta. The tree possesses therapeutic potential (Panda et al. 2015) and ethnobotanical (Singh and Pandey 1998) properties. Natural population of the tree is decreasing due to physiological obstacles such as seed coat imposed dormancy (Prasad and Nautiyal 1996) and high seedling mortality by drying off the radical (Ravindranath et al. 2003). No alternative means for the propagation have been developed yet. Therefore, a micropropagation system involving axillary shoot proliferation through mature tree derived nodal explants in *B. racemosa* was developed by our group (Sharma et al. 2017) recently. In continuation of the developed protocol, genetic stability of micropropagated plants developed by Sharma et al. (2017) was evaluated using two gene targeted markers i.e. CBDP and SCoT in the present study. This is the first report on the application of SCoT and CBDP markers for analysis of genetic stability of micropropagated plants of *B. racemosa*.

Materials and methods

Plant material and extraction of genomic DNA

Juvenile leaves were collected from nine plants of *B. racemosa* randomly selected from the population of micropropagated plants established by using protocol developed by Sharma et al. (2017) (Fig. 1a–c). The selected micropropagated plants of 1 year old successfully established in field were subjected to molecular analysis. Leaf samples were also taken from the donor tree from which explants were collected for micropropagation. The samples were fixed in liquid nitrogen (− 196 °C) and ground to fine powder using sterile pestle and mortar. In order to reduce the effect of phenolics on DNA extraction, these samples were subjected to wash with freshly prepared triton solution as described by Gupta et al. (2011). Modified CTAB method (Doyle and Doyle 1990) was used for extraction of DNA from triton washed samples and further purification was performed using phenol washing method (Murray and Thompson 1980). Quality and quantity of extracted DNA were assayed spectrophotometrically (UV–Visible Elico spectrophotometer) by the A260/A280 absorbance ratio. The integrity of the DNA was analyzed by electrophoresing the DNA on 0.8% (w/v) agarose gel (A9539, Sigma, India) in 1X TBE buffer.

CBDP-PCR analysis

After preliminary screening of 25 CBDP primers (designed by Singh et al. 2014), we selected 21 primers (Table 1) for genetic fidelity analysis of micropropagated *B. racemosa*. Each PCR reaction was prepared in a total volume of 15 µl which contained 2 µl of template DNA (50–60 ng), 1.5 µl of 10 × PCR buffer [(100 mM Tris (pH 9.0), 500 mM KCl, and 1% Triton X-100 with 15 mM $MgCl_2$)] (Sigma, India), 1.5 µl $MgCl_2$ (2.5 mM) (Sigma, India), 0.4 µl of dNTPs (10 mM; Sigma, India), 1.5 µl of each primer, 0.5 µl of three-unit Taq polymerase (Sigma, India) and 7.6 µl sterile nuclease free water (Genetix Biotech). PCR conditions for CBDP primers were as follows: Initial denaturation for 5 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 1 min; annealing at T_a °C

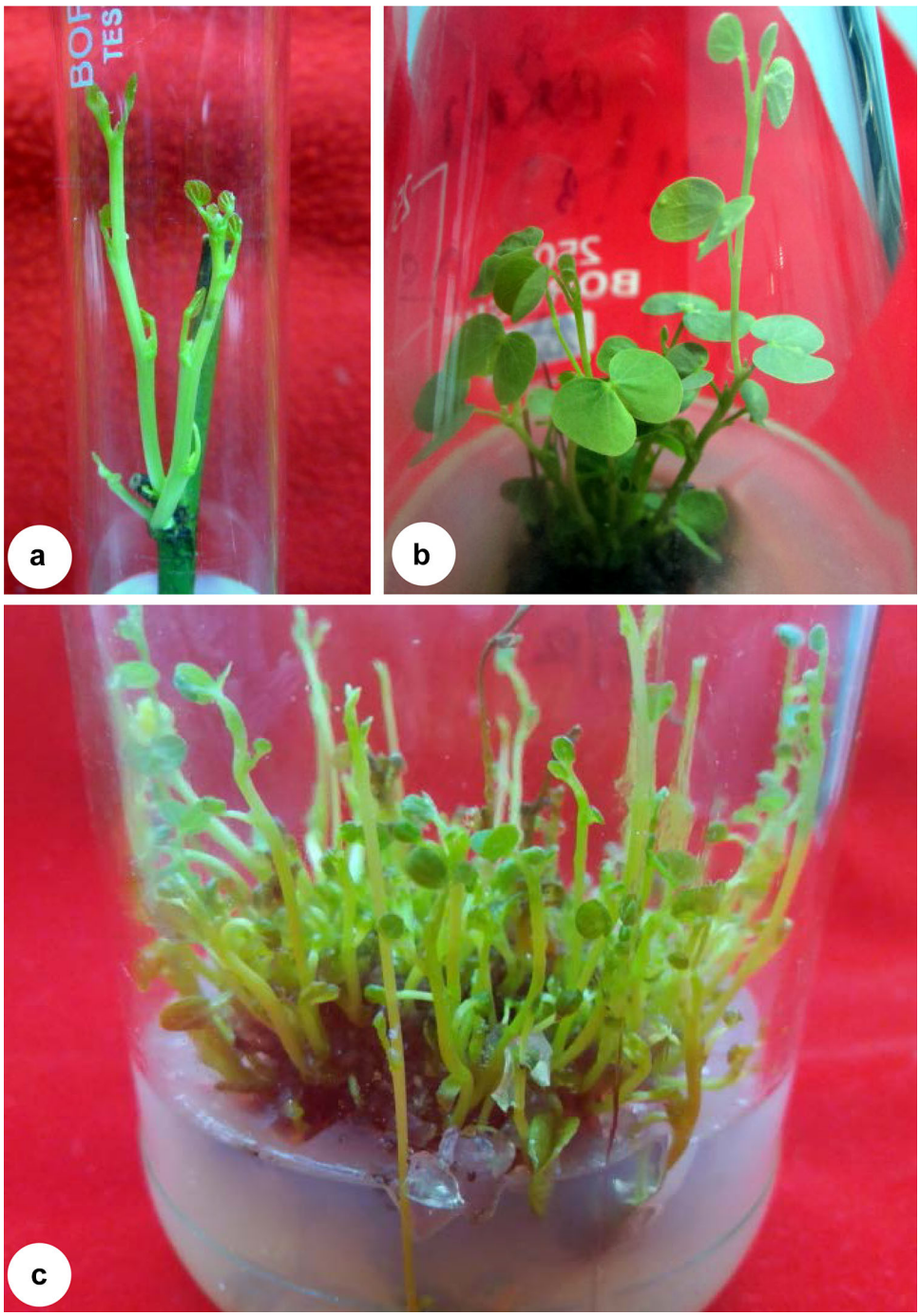

Fig. 1 Micropropagation of *B. racemosa* involving axillary shoot proliferation from mature tree derived nodal explants. **a** Axillary shoot induction, **b** shoot clump formation and **c** multiple shoot proliferation

(54.8–63.6) for 1 min and extension at 72 °C for 1 min. A final extension of 10 min at 72 °C was carried out.

SCoT-PCR analysis

A set of 18 SCoT primers (developed by Collard and Mackill 2009, Table 2) was selected after preliminary screening of 36 primers for genetic stability assessment. A total volume of 15 µl reaction mixture was prepared as mentioned earlier for CBDP marker. PCR programme were as follows: Initial denaturation at 94 °C for 4 min; 38 cycles of denaturation at 94 °C for 30 s; annealing at T_a °C (52.3–58.9) for 1 min and extension for 1 min at 72 °C. This was followed by a final extension at 72 °C for 10 min.

Visualization of CBDP and SCoT products

The amplified products were stored at 4 °C until further analysis. PCR product separation gels were made of 1.4% (w/v) agarose (A9539, Sigma, India) in 1X TBE buffer and

Table 1 List of CBDP primer sequences with the number and size range of scorable amplified fragments produced in *B. racemosa* mother plant and micropropagated plants

Sr. no.	Primer code	Primer sequence (5′–3′)	T_a	Number of scorable/monomorphic bands	Size range of amplification products (bp)
1	CAAT 1	TGAGCACGATCCAATAGC	60.0	5/5	300–1100
2	CAAT 2	TGAGCACGATCCAATAAT	56.4	4/4	550–1400
3	CAAT 3	TGAGCACGATCCAATACC	59.6	6/6	300–1300
4	CAAT 4	TGAGCACGATCCAATAAG	57.2	3/3	450–1300
5	CAAT 5	TGAGCACGATCCAATCTA	57.7	6/6	375–1250
6	CAAT 6	TGAGCACGATCCAATCAG	61.1	6/6	225–1350
7	CAAT 9	TGAGCACGATCCAATGAT	60.2	4/4	250–700
8	CAAT 10	TGAGCACGATCCAATGTT	60.3	2/2	850–900
9	CAAT 11	TGAGCACGATCCAATTGC	63.6	6/6	650–1450
10	CAAT 12	TGAGCACGATCCAATATA	54.8	3/3	600–1000
11	CAAT 15	TGAGCACGATCCAATTGA	61.5	4/4	500–1000
12	CAAT 16	TGAGCACGATCCAATTCA	61.5	8/8	200–2000
13	CAAT 17	TGAGCACGATCCAATTTG	60.9	4/4	700–1100
14	CAAT 18	CTGAGCACGATCCAATAG	56.7	5/5	350–1400
15	CAAT 19	CTGAGCACGATCCAATAC	56.5	4/4	350–600
16	CAAT 20	CTGAGCACGATCCAATAT	55.9	6/6	400–1000
17	CAAT 21	CTGAGCACGATCCAATCA	61.1	6/6	275–1200
18	CAAT 22	CTGAGCACGATCCAATCG	63.2	3/3	300–1300
19	CAAT 23	CTGAGCACGATCCAATGG	63.1	6/6	500–1400
20	CAAT 24	CTGAGCACGATCCAATGA	61.1	3/3	850–1250
21	CAAT 25	CTGAGCACGATCCAATGT	59.8	3/3	650–1100

T_a annealing temperature

stained with ethidium bromide (20 µl/100 ml gel; Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India). Amplified DNA fragments were resolved at a stable voltage of 150 V for 2 h in 1X TBE buffer using submerged gel electrophoresis (GeNei^{TM}, Bangalore, India) and then picturized under the gel documentation system (Syngene Gel Doc, Syngene, Synoptics Ltd., UK). To determine the size of amplified bands, 100 bp DNA ladder (MBT049, Himedia, India) was used as size marker.

Data scoring and analysis

In order to confirm the consistency of results, all the experiments were conducted twice. Only consistent, well resolved and unambiguous fragments in the size range of 100 bp to 3000 bp were scored manually. Each band was treated as a marker. For the purpose of scoring, all markers were treated as dominant and scored on the basis of presence (1) or absence (0) in the gel. The DNA bands present at low intensity which could not be readily distinguished as either present or absent were considered as ambiguous markers and were not scored.

Results and discussion

In the present study, two gene targeted markers i.e. CBDP and SCoT were employed for evaluating genetic stability of micropropagated plants of *B. racemosa*.

A total of 21 CBDP primers produced 97 scorable DNA fragments with an average of 4.61 bands per primer. Amplification products ranged in size from 225 to 2000 bp and the number of bands per primer varied from 2 (CAAT 10) to 8 (CAAT 16) (Table 1). Monomorphic banding pattern obtained from CBDP (CAAT 20) marker is shown in Fig. 2a. The amplified products were observed as monomorphic in all micropropagated and mother plant confirming the genetic homogeneity.

CBDP is a novel promoter-targeted marker system which exploits the nucleotide sequence of CAAT box region of the promoter in plant genes. CBDP primers contain 18 nucleotides sequence including a CCAAT nucleotides core which is flanked by the filler sequence (5′ end) and di- or trinucleotides sequence (3′ end) (Singh et al. 2014). Singh et al. (2014) validated CBDP marker in cotton (*Gossypium* species), jute (*Corchorus capsularis* and *C. oitorius*) and linseed (*Linum usitatissimum*)

Table 2 List of SCoT primer sequences with the number and size range of scorable amplified fragments produced in *B. racemosa* mother plant and micropropagated plants

Sr. no.	Primer code	Primer sequence (5′–3′)	T_a	Number of scorable/monomorphic bands	Size range of amplification products (bp)
1	SCoT 1	CAACAATGGCTACCACCA	52.6	5/5	400–1400
2	SCoT 2	CAACAATGGCTACCACCC	53.6	4/4	500–1200
3	SCoT 3	CAACAATGGCTACCACCG	53.9	8/8	250–2500
4	SCoT 4	CAACAATGGCTACCACCT	52.3	5/5	600–2000
5	SCoT 5	CAACAATGGCTACCACGA	52.6	2/2	500–1300
6	SCoT 6	CAACAATGGCTACCACGC	54.4	7/7	400–1300
7	SCoT 7	CAACAATGGCTACCACGG	53.9	2/2	600–1000
8	SCoT 8	CAACAATGGCTACCACGT	52.9	5/5	550–1200
9	SCoT 9	CAACAATGGCTACCAGCA	52.9	3/3	750–950
10	SCoT 11	AAGCAATGGCTACCACCA	54.4	11/11	275–1700
11	SCoT 12	ACGACATGGCGACCAACG	58.4	5/5	550–1400
12	SCoT 13	ACGACATGGCGACCATCG	58.0	4/4	320–800
13	SCoT 16	ACCATGGCTACCACCGAC	57.3	5/5	500–900
14	SCoT 24	CACCATGGCTACCACCAT	54.8	6/6	250–1800
15	SCoT 26	ACCATGGCTACCACCGTC	57.3	6/6	300–2000
16	SCoT 27	ACCATGGCTACCACCGTG	57.6	2/2	550–700
17	SCoT 33	CCATGGCTACCACCGCAG	58.9	3/3	450–800
18	SCoT 36	CGCAACAATGGCTACCACC	54.2	5/5	350–900

cultivars. Recently, CBDP markers have been used for analysis of genetic diversity in Jojoba (Heikrujam et al. 2015) and *Andrographis paniculata* (Tiwari et al. 2016). However, there is no published report on the application of CBDP markers in detecting genetic fidelity of micropropagated plants.

SCoT is a simple and reliable dominant marker and unlike RAPD or ISSR which are based on non coding regions of genome, SCoT technique is correlated to functional genes as well as their corresponding traits (Xiong et al. 2011). Multilocus nature of the marker is helpful in scoring high genetic polymorphism. In addition, use of long primers and high annealing temperature enhance the reproducibility of SCoT primers (Xiong et al. 2011). Being associated with initiation codon; SCoT markers are abundant in genome and provide extensive genetic information (Chhajer et al. 2017).

In the present study, a total of 18 SCoT primers produced 88 scorable DNA fragments ranging in size from 250 to 2500 bp (Table 2). The average of DNA bands per primer was 4.88. The number of amplicons per primer varied from 2 (SCoT 5, SCoT 7 and SCoT 27) to 11 (SCoT 11) bands. Similar to CBDP marker, the amplicons observed for SCoT marker were also monomorphic in nine micropropagated plants as well as the mother plant. Figure 2b shows monomorphic banding patterns obtained from SCoT (SCoT -11).

Use of SCoT markers to assess the genetic stability was first reported by Rathore et al. (2014) in *Cleome gynandra* and later by several other authors in various plants (Agarwal et al. 2015; Chhajer and Kalia 2016; Sathish et al. 2018) revealing the monomorphic band pattern as reported in present study. Rathore et al. (2014) reported 2–7 amplified fragments (average 4.3 bands per primer) in the size range of 200–1500 bp while more recently Sathish et al. (2018) reported 3–9 fragments (average 5.25 bands per primer) with the size range of 200–2000 bp in *Saccharum officinarum*. Besides, SCoT system has been successfully utilized in other aspects of plant studies such as genetic diversity assessment, mapping of quantitative trait loci (QTL), DNA fingerprinting, identification of cultivars, analysis of male and female genotypes, bulk segregation analysis, development of co-dominant SCAR (Sequence characterized amplified region) markers (Collard and Mackill 2009; Gorji et al. 2012; Cabo et al. 2014; Heikrujam et al. 2015; Chhajer et al. 2017; Feng et al. 2018; Shekhawat et al. 2018).

The results generated in the study corroborate the true to type nature of micropropagated plants and authenticate that *B. racemosa* in vitro cultures developed according to the described protocol (Sharma et al. 2017) are free from somaclonal variations. They also support the view that development of in vitro cultures through organized primordia (pre-existing meristems) especially apical

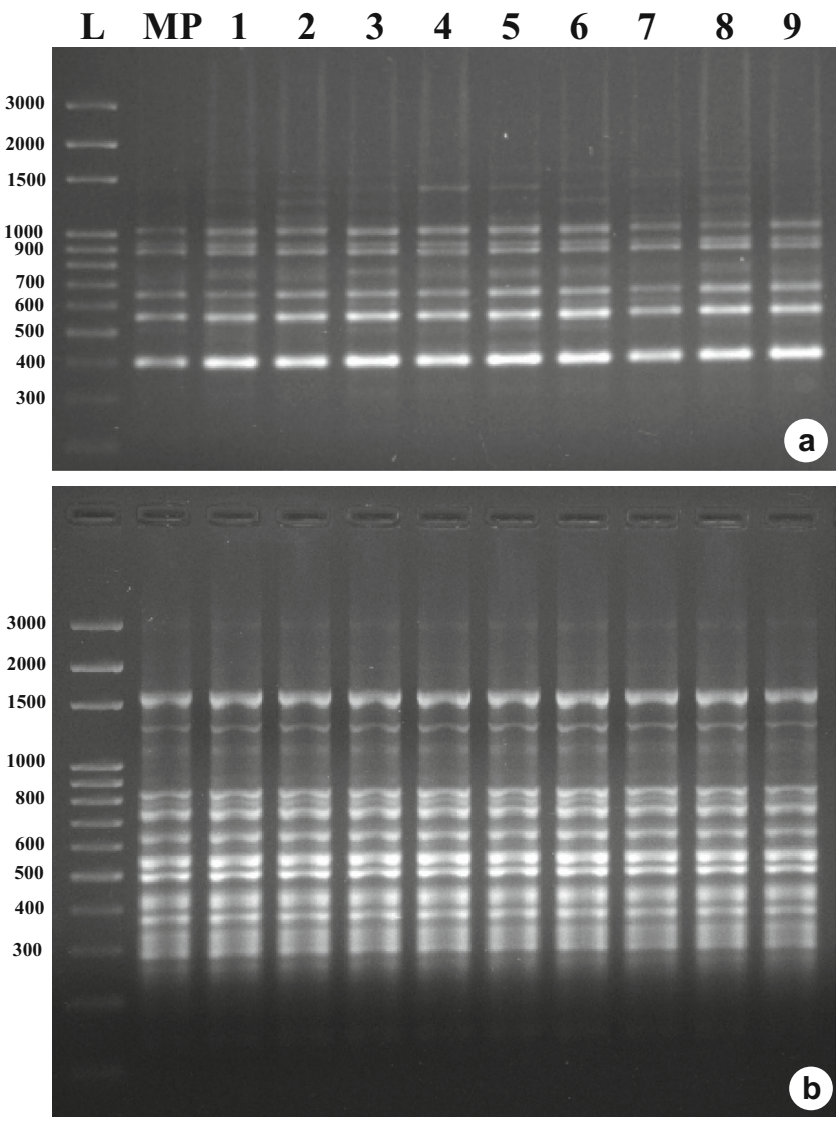

Fig. 2 Validation of genetic homogeneity in micropropagated plants of *B. racemosa* and their corresponding mother plant. DNA fingerprinting profile obtained through **a** CBDP (CAAT-20) and **b** SCoT (SCoT -11) markers. *Lanes*: L 100 bp DNA Ladder; MP Mother Plant; 1–9 Micropropagated plants transferred to the field

meristems and axillary buds is a reliable method of producing genetically stable plant population as the tissue culture of organized meristems circumvent the dedifferentiation and/or redifferentiation of cells or tissues (Negi and Saxena 2010; Phulwaria et al. 2013).

In conclusion, gene targeted markers (CBDP and SCoT) were used for clonality assessment of micropropagated *B. racemosa*. True to type nature of micropropagated plants was authenticated as the amplified bands were monomorphic in nature. The present study supports the view that organized meristem cultures are genetically more stable than callus derived regeneration (Phulwaria et al. 2013). Thus the developed in vitro propagation protocol from mature tree derived nodal explants can be utilized for mass multiplication of *B. racemosa.* This is the first report on exploring the genetic stability of micropropagated *B. racemosa* using molecular markers and also the first study reporting the use of CBDP markers for genetic stability analysis of any micropropagated plant.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

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