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Detection of somaclonal variants using RAPD marker in *Bacopa monnieri* and *Tylophora indica*

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Abstract Random amplified polymorphic DNA (RAPD) marker was used to detect somaclonal variants between in vivo and in vitro plants of *Bacopa monnieri* and *Tylophora indica*. Ten random decamer primers were used and out of them 9 primers gave reproducible results for both the plants. Total 58 amplified products were observed for both the plants and the size of these amplified products ranges from 150 bp to 1250 bp, 50 bp to 1200 bp for *B. monnieri* and *T. indica* respectively. An average polymorphism was found to be 13.19% for *B. monnieri* and 62.07% for *T. indica*.

Key words: *Bacopa monnieri*; leaf explants; RAPD; somaclonal variation; *Tylophora indica*.
Abbreviations: BAP, N_6- Benzyl amino purine; bp, base pair; Kn, Kinetin; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA.

Introduction

Bacopa monnieri (L.) Wettst (Scrophulariaceae) and *Tylophora indica* (Burm. F.) Merrill (Asclepiadaceae) are important medicinal plants. *B. monnieri* contains a number of biologically active compounds such as des-saponin glycosides-triterpenoid saponins (Bacosides A & B). *B. monnieri* is used in treatment of anxiety and neurosis (Singh *et al.*, 1980). Other uses of this plant are as an analgesic, antipyretic, anti-inflammatory and memory vitalizer (Anonymous, 1988; Agrawal, 1993; Vohora *et al.*, 1997). *T. indica* contains alkaloids such as tylophorine, tylophorinine and tylophorinidine (Mulchandani *et al.*, 1971; Rao *et al.*, 1971; Chandrasekhar *et al.*, 2006). This plant has been used in the treatment of asthma and bronchitis (Gupta *et al.*, 1979; Singh *et al.*, 2009). It is also reported to have antitumor, immunosuppressive and anti-

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inflammatory properties (Donaldson *et al.*, 1968, Gopalakrishnan *et al.*, 1980). Ever increasing demands of these plants has lead to rapid depletion from wild and techniques like plant tissue culture have helped in *ex vitro* conservation and rapid multiplication of the species (Pandey *et al.*, 1993; Faisal *et al.*, 2005).

In vitro propagation techniques are known to generate somaclonal variations (Larkin and Scowcroft, 1981; Chuang *et al.*, 2009) and hence it is important and mandatory to test the genetic homogeneity of plants. Variants with desired characters can be useful when secondary metabolite content is increased as observed in *Hypericum perforatum* (Cellárová *et al.*, 1994). These variations can be determined at the morphological, cytological, biochemical and molecular levels, but morphological variations occur at much lower frequency (Evans *et al.*, 1984) and can be detected only when a plant matures (Mahmood *et al.*, 2010). They are also influenced by environmental factors and may not reflect the true genetic composition of the plant (Mandal *et al.*, 2001).

Similarly biochemical tests for detection of somaclonal variations are complex and require high expertise. In comparison molecular techniques are considered to be valuable tools for detecting genetic fidelity of *in vitro* plants at an early stage (Bairu *et al.*, 2011). Molecular markers are suitable and effective in assessing the genetic stability as these are not influenced by environmental factors (Kawiak and Lojkowska, 2004; Kumar *et al.*, 2009). RAPD is one such marker which is widely used as it is sensitive and useful in detection of the variants (Williams *et al.* 1993; Bordallo *et al.*, 2004; Chuang *et al.*, 2009). This method is simple, relatively cheap, quick in performance, requires less amount of DNA and can be carried out without any prior knowledge of target genome (Welsh and Mc Clelland, 1990; Williams *et al.*, 1990). It is a PCR based technique in which enzymatic amplification of DNA with arbitrary primers results in several discrete DNA fragments (Kirtikar and Basu, 1990; Kumar *et al.*, 2009). Polymorphism is due to the change in nucleotide base which alters the primer binding site as well as insertion or deletion within the amplified region. This results in presence or absence of amplification products from different locus/loci (Tingey and del Tufo, 1993).

The present study was conducted for detecting somaclonal variants in *B. monnieri* and *T. indica* plantlets developed through leaf explants.

Materials and methods

The leaf explants of *B. monnieri* and *T. Indica* were collected from young healthy vegetative plants growing in the Botanical Garden of the M. S. University of Baroda and used for establishment of cultures. Murashige and Skoog's (MS) (1962) medium supplemented with sucrose (3%) and BAP (6 µM) (SRL, Mumbai, India) (Joshi *et al.*, 2010) was used for *B. monnieri* shoot

cultures. Similarly in *T. indica* the MS medium was fortified with sucrose (3%) along with BAP (4 µM) and Kn (8 µM) (SRL, Mumbai, India) (*Unpublished data*) for developing shoot cultures.

For RAPD analysis, wild and *in vitro* grown leaves of both the plants were used for extraction of DNA as it is relatively easy for acquisition and preparation (Jarret, 1986). The extraction of DNA was done according to the method standardized for these plants (Pathak *et al.*, 2013). Random decamer primers with 60-70% GC content was ordered and purchased (Eurofins Genomics India, Bangalore, India) and sequences of primers are as follows: 5'-GTGATCGCAG-3', 5'-CCACAGCAGT-3', 5'-ACCCCCGAAG-3', 5'-GGACCCTTAC-3', 5'-TTGGCACGGG-3', 5'-GTGTGCCCCA-3', 5'-TGGACCGGTC-3', 5'-GGGTAACGCC-3', 5'-GTGATCGCAT-3', 5'-AGCCAGCGAA-3' (Henceforth RP1 to RP10 respectively).

For PCR, reaction mixture of the total volume of 20 µL included 2 µL (100 ng) of genomic DNA, 12 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 2 µL of 10 µM primer (Eurofins, Bangalore, India) and 0.2 µL (5 U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 94℃ for 5 min followed by 45 cycles of 94℃ for 1 min, 37℃ for 1 min, 72℃ for 2 min and final extension at 72℃ for 10 min. The amplified products were checked by electrophoresis on 1.2% agarose gel stained with ethidium bromide.

Results and discussion

In the present study, a total 10 arbitrary primers were screened to assess the genetic fidelity of the *in vitro* shoots, regenerated from the leaf explant. Arbitrary primers used in the present study, the total number of bands scored, their bp size with % polymorphism for each primer in both the plants is given in Table 1.

Profiles of the amplified RAPD markers for the two species showed that wild plant and *in vitro* had different banding patterns of amplified DNA (Fig 1 and 2). In *B. monnieri*, 9 out of 10 primers gave reproducible results of total 58 amplification products in the size range of 150 to 1250 bp. The number of amplified products generated per primer varied from 4 to 8, of which primers RP6, RP8 and RP9 produced maximum bands and RP4 gave minimum bands.

Similarly for *T. indica*, same 9 primers gave a total of 58 products within the size range from 50 to 1200 bp, with the number of bands varied from 4 (RP9) to 9 (RP5). An average 6.44 bands per primer were produced for both *B. monnieri* and *T. indica* (Table 1).

In case of *B. monnieri*, total 8 products were found to be polymorphic (13.19%) and 50 products were monomorphic (86.21%). Primer RP9 generated maximum of 4 while RP3, RP5, RP8 and RP10 primers produced minimum 1 polymorphic band and for monomorphic bands primer RP6 produced maximum (8) and RP4 and RP9 produce minimum (4) bands. Whereas in *T. indica*, total 36 products were found to be polymorphic (62.07%) and 22 were monomorphic (37.93%). A maximum of 7 and minimum 1 polymorphic bands were generated by primers RP4 and RP3 and RP10 respectively; whereas primer RP10 produced maximum (5) and RP4 produced minimum (1) monomorphic bands. The highest polymorphism was found in case of primer RP9 (50%) for *B. monnieri* and primers RP8 and RP9 (100%) for *T. indica* (Table1). The bands showing polymorphism are marked by black arrows (Fig. 1 and 2).

Table 1. RAPD profile of *B. monnieri* and *T. indica in vitro* shoots using random primers

Primer	Sequence (5'-3')	Amplicon size range (bp)		Polymorphic bands/total no. of bands		% Polymorphism	
		B. monnieri	*T. indica*	*B. monnieri*	*T. indica*	*B. monnieri*	*T. indica*
RP1	GTGATCGCAG	150-500	50-950	0/5	2/5	0	40
RP2	CCACAGCAGT	310-1100	200-850	0/6	4/7	0	57.14
RP3	ACCCCCGAAG	150-1150	250-600	1/6	1/5	16.67	20
RP4	GGACCCTTAC	400-1150	200-1000	0/4	7/8	0	87.5
RP5	TTGGCACGGG	200-1250	200-1000	1/7	5/9	14.28	55.55
RP6	GTGTGCCCCA	180-1150	250-900	0/8	6/8	0	75
RP7	TGGACCGGTC	-	-	-	-	-	-
RP8	GGGTAACGCC	150-1150	280-1150	1/8	6/6	12.50	100
RP9	GTGATCGCAT	150-1150	250-1200	4/8	4/4	50	100
RP10	AGCCAGCGAA	300-700	200-1050	1/6	1/6	16.67	16.67

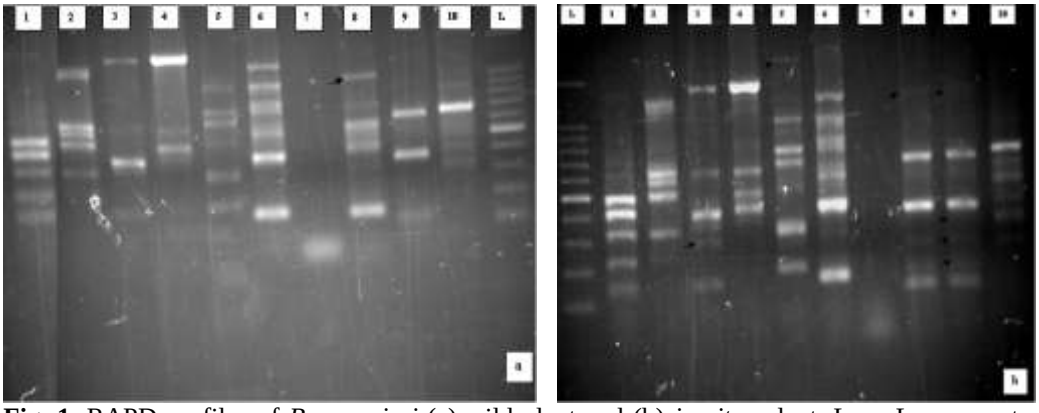

Fig. 1. RAPD profiles of *B. monnieri* (a) wild plant and (b) *in vitro* plant. Lane L represents 100 bp ladder and Lane 1 to 10 represent amplification of DNA with primers RP1 to RP10 respectively.

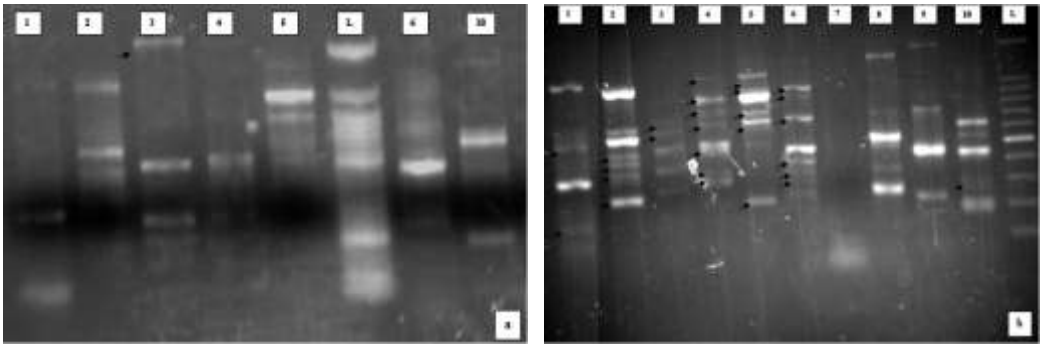

Fig. 2. RAPD profiles of *T. indica* (a) wild plant and (b) *in vitro* plant. Lane L represents 100 bp ladder and Lane 1 to 10 represent amplification of DNA with primers RP1 to RP10 respectively

The gel pattern of *in vivo* and *in vitro B. monnieri* plants depicted gaining bands except for the band of 950 bp which was absent in case of *in vitro* plant for primer RP8. Similarly for *T. indica*, primer RP3 gave band of 1.25 kb which was observed only in mother plant and absent from *in vitro* plant; whereas other bands were gained in *in vitro* plant. Presence of a band in both plants (i.e. wild and *in vitro* plant) indicates a high level of sequence homology at that site (Williams *et al.*, 1990), whereas in case of absence of band from one but not the other indicates there may be difference in sequence (Hashmi *et al.*, 1997).

These variations which are known to occur in tissue culture plants can be either pre-existing or induced and the potent source of pre-existing variations is known as chimeras (George, 1993). Whereas tissue culture induced variation include mode of regeneration, type of explant, type and concentration of the plant growth regulators as well as number and duration of subculture (Bairu *et al.*, 2011). One reason for occurrence of polymorphism in the present study may be due to indirect regeneration of the plantlets and in *Pelargonium* spp. similar findings are reported stating that the variability is greater in indirect organogenesis as compared to direct regeneration (Skirvin and Janick, 1976).

As leaf was the source of explant for regeneration in both the plants, there are chances for development of somaclonal variations. There are several reports which have shown occurrence of genetic variation in plants regenerated through leaf tissue e.g. *Ipomea batatas* (Munthali *et al.*, 1996), *Drosera anglica* (Kawiak and Łojkowska, 2004) and *Silybum marianum* (Mahmood *et al.*, 2010). Another possible reason for induction of genetic variation is due to *in vitro* stress faced by the plants (Larkin and Scowcroft, 1981; Evans *et al.* 1984).

Using RAPD marker for detecting variants in *in vitro* plants has been reported earlier in *B. monnieri* and *T. indica* (Jayanthi and Mandal, 2001; Ceasar *et al.*, 2010; Ramesh *et al.*, 2011). Similar findings have been observed in *in vitro* plants regenerated from different explants like leaf sheath in

Curcuma longa (Salvi *et al.*, 2001); *Curcuma amada* (Prakash *et al.*, 2004); from shoot tip in *Phoenix dactylifera* (Eshraghi *et al.* 2005), bud flower stalks in *Phalaenopsis* (Chen *et al.*, 1998); meristem culture in *Melia azedarach* (Olmos *et al.*, 2002).

These results indicate that there was a variation among the *in vivo* and *in vitro* plants of *B. monnieri* and *T. indica*. As somaclonal variants can become a source for improved secondary metabolite yield (Thomas et al. 2006; Rana *et al.* 2012), further studies in this aspect can be taken up for these two medicinally important plants. The present study was an initial step towards the identification of variants and it was concluded that RAPD markers could detect somaclonal variants in *B. monnieri* and *T. indica* regenerated from the leaf explants.

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