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Research article

Genetic analysis of micro propagated plants of *Tinospora cordifolia* (Willd.) Miers by using RAPD–PCR markers

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Abstract: Random Amplified Polymorphic DNA (RAPD) is the simplest and cost-effective tool used for the analysis of the genetic fidelity of *in vitro* propagated plants of *Tinospora cordifolia*. A high-quality genomic DNA free from any contaminants, was isolated from five genotypes by the modified CTAB method. Out of 30 random decamer primers, OPA-02, OPA-03, OPA-09, OPA-10, OPB-02, OPB- 04, OPB-09 and OPK-06 showed fingerprints. Out of total 27 scored bands, 26 produced were monomorphic bands (96.2%) and one was polymorphic band (3.8%). The highest number of bands (6) were produced by OPA -03 and OPK-06 and minimum number of bands (1) was produced by OPB -04. The amplicon size varied from 200 bp to 1500 bp. A coefficient similarity value ranging from 0.87–0.93 was obtained. Maximum similarity value 0.93 was exhibited by both the genotypes namely, SB1 and SB3. From the present study, it has been concluded that the RAPD analysis can be used to check the genetic fidelity in the micro propagated plants of *Tinospora cordifolia*.

Keywords: Random primers - *Tinospora cordifolia* - Molecular analysis - *in vitro* regenerated.

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INTRODUCTION

Tinospora cordifolia (Willd.) Miers is a large glabrous, deciduous climbing shrub belonging to the family Menispermaceae. It is commonly named as "Guduchi". It is widely used in veterinary folk medicine and ayurvedic system of medicine because of possessing anti-periodic, antispasmodic, anti-inflammatory, anti-arthritic, anti-allergic and anti-diabetic properties (Saha & Ghosh 2012). It particularly helps in the treatment of diseases like fever, jaundice, general debility, cough, asthma, leucorrhoea, skin diseases, bites of poisonous insects and venomous snakes and eye disorders (Singh *et al.* 2003). Tissue culture has greatly enhanced the scope and potential of mass propagation by exploiting the regenerative behavior in a wide range of selected horticultural and agricultural plants including medicinal ones. Many important medicinal herbs throughout the world have been successfully propagated in vitro, either by organogenesis or by callus formation Plant tissue culture is a reliable technique used to produce true-to-types plants. During plant tissue culture, there are chances of occurrence of somaclonal variations so the plants obtained through micropropagation must be screened for its clonal fidelity.

The utility of molecular analysis to check the genetic fidelity of *in vitro* regenerated plants has been well documented by many workers (Yadav *et al.* 2004, Khawale *et al.* 2006, Samantaray & Maiti 2010, Senapati *et al.* 2013, Saha *et al.* 2014, Borsai *et al.* 2020). RAPD (Random Amplified Polymorphic DNA) have been proven extremely useful in determining the genetic stability of micro-propagated plants because of less DNA is required, ease of use, low cost, reliability and do not require pre-knowledge of the sequence of plants under study (Williams *et al*. 1990). They are an inexpensive and versatile set of markers that rely upon repeatable amplification of DNA sequence using primers (Khan *et al*. 2009, Khan *et al.* 2011, Kaur & Neetu 2013, Neetu *et al*. 2017).

MATERIALS AND METHODS

Source of material: Isolation of DNA was done by using leaves of mother stock designated as SB1 and

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micro propagated plants (from forth subculture to seventh subculture) designated as SB2 to SB5 of *Tinospora cordifolia*. Motherstock plants of *Tinospora cordifolia* were taken from naturally growing plants collected from sector 32, Chandigarh and maintained at the Department of Biotechnology, Goswami Ganesh Dutta Sanatan Dharma College, Chandigarh, India for further micropropagation studies.

Isolation of DNA

In the present study, DNA isolation and purification were done by modified CTAB protocol given by Doyle & Doyle (1990). Quantification of DNA is done by agarose gel electrophoresis on 1% (w/v) agarose gel with a known amount of lambda DNA marker (MBI, Fermentas, Richlands B.C. Old). The DNA was then diluted in TE buffer to 25 ng μl^{-1} concentration for further use in a polymerase chain reaction (PCR). The RAPD reaction was performed according to the method developed by (Welsh & McClelland 1990). A set of 30 random decamer primers of series OPA-01 to OPA-10, OPB-01 to OPB-10 and OPK-01 to OPK-10 were purchased from Operon technologies to be used as a single primer for the amplification of RAPD fragments. RAPD Primers were screened for the presence of monomorphic and distinct bands. Each reaction tube contained 50 ng template DNA, $MgCl_2$ (100 mM), dNTPs (100 mM), 3 U μl^{-1} of Taq DNA polymerase (Sigma Aldrich, USA) and PCR buffer. The amplification was performed in a DNA thermal cycler (Bio-Rad) using the following PCR programme :Complete denaturation at 94°C for 5 mins followed by 45 cycles at 94°C for 1 min, 36°C for 30 sec and 72°C for 1 min, final extension at 72°C for 2 mins. PCR Reaction is stopped by adding DNA loading dye and PCR products were resolved in 1% agarose gel in 1X TBE buffer with 2 kb molecular weight marker. The PCR amplified products were visualized and photographed under a U.V. transilluminator. The electrophoretic profile was analyzed for polymorphism based on the presence and absence of DNA bands on agarose gel. The sizes of DNA fragments were estimated by comparison with standard ladder (2 kb, Sigma Aldrich, USA).

RAPD Data analysis

The gel pictures acquired through gel documentation system into the computer were processed and scored to obtain binary data. The amplified products were recorded as present (1) or absent (0) for the individual genotypes. The similarity index was estimated using Jaccard's similarity indices. Cluster analysis was carried out on similarity estimates using the UPGMA method using NTSYS pc, version 2.0 (Rohlf 2000).

RESULTS AND DISCUSSION

DNA based molecular markers have emerged as a powerful technique for the determination of genetic relatedness and therefore are being used in many crops. RAPD technique has been successfully employed by many authors to confirm genetic uniformity among tissue culture raised plants (Bairu *et al*. 2011). In this work, the genetic stability of the regenerated plants was screened by RAPD markers The DNA samples extracted from five genotypes of *Tinospora cordifolia* were pure and of a great quality. For accuracy of the results, the high quality and purity of genomic DNA free from any contaminants i.e secondary metabolites, proteins and RNA was isolated from these genotypes by using modified CTAB method. For any RAPD reaction to perform , it was necessary to standardise the variables used for the successful and clear amplification of DNA . RAPD primers donot amplify the DNA below a certain amount. DNA quantifications were performed by visualizing gel under U.V. light, after electrophoresis on 0.8% agarose gel at 60 volt cm^{-1} for 2 hours with a known amount of lambda DNA marker.

Out of 30 random decamer primers, Eight random decamer of series OPA-02, OPA-03, OPA-09, OPA-10, OPB-02, OPB- 04, OPB-09 and OPK-06 showed fingerprints. These were selected to reveal the genetic similarity among the *Tinospora cordifolia* samples. All the eight gels obtained from the above short-listed primers had given the maximum number of clear and scorable amplicons in DNA sample. The highest number of bands (6) were produced by OPA-03 and OPK-06 and minimum number of bands (1) was produced by OPB -04 (Table 1). The amplicon size varied from 200 bp to 1500 bp (Table 2).

Table 1. Total number of amplified DNA fragments and polymorphic DNA fragments generated by PCR using eight random decamer primers of *Tinospora cordifolia* (Willd.) Miers plants.

Primers	Total Scored Bands	Polymorphic Bands	Monomorphic Bands
OPA-02	5	0	5
OPA-03	6	1	5
OPA-09	2	0	2
OPA-10	3	0	3
OPB-02	3	0	3

OPB-04	1	0	1
OPB-09	2	0	2
OPK-06	6	0	6

Table 2. Amplified DNA product size range generated by eight random decamer primers in different samples of *Tinospora cordifolia* (Willd.) Miers.

Primers	Amplified Product Size Range (Base pair)
OPA-02	300-1500
OPA-03	300-1500
OPA-09	400-1500
OPA-10	500-1500
OPB-02	300-1200
OPB-04	200-1500
OPB-09	200-800
OPK-06	300-1500

Out of total 27 scored bands, 26 produced were monomorphic bands (96.2%) and one was polymorphic band (3.8%). It mean almost all banding profiles from micropropagation plants were monomorphic and similar to those of the mother plant. On a similar note, Chandrika *et al*. (2008) reported that out of total 183 bands scored, 178 bands produced were monomorphic (97.2%) and five bands were polymorphic (2.73%) among the 21 in *vitro grown* plants of *Dictyospermum ovalifolium* Wight. Khatab & Youssef (2018) generated 38 bands from ten RAPD primers which were monomorphic among the micropropagated plants of *Musa* sp. cv. Williams compared to the mother plant; except one combination of primers have generated only one polymorphic band. Similarly for confirming the genetic stability among micropropagated and the mother plants of *Inula royleana* DC, molecular markers RAPD has given monomorphic banding pattern (Amin *et al*. 2018). No polymorphism in *in vitro* propagated plants and mother plant of blackberry was reported by Borsai *et al*. (2020). All monomorphic bands were observed which proved the genetic fidelity of micro-propagated plants.

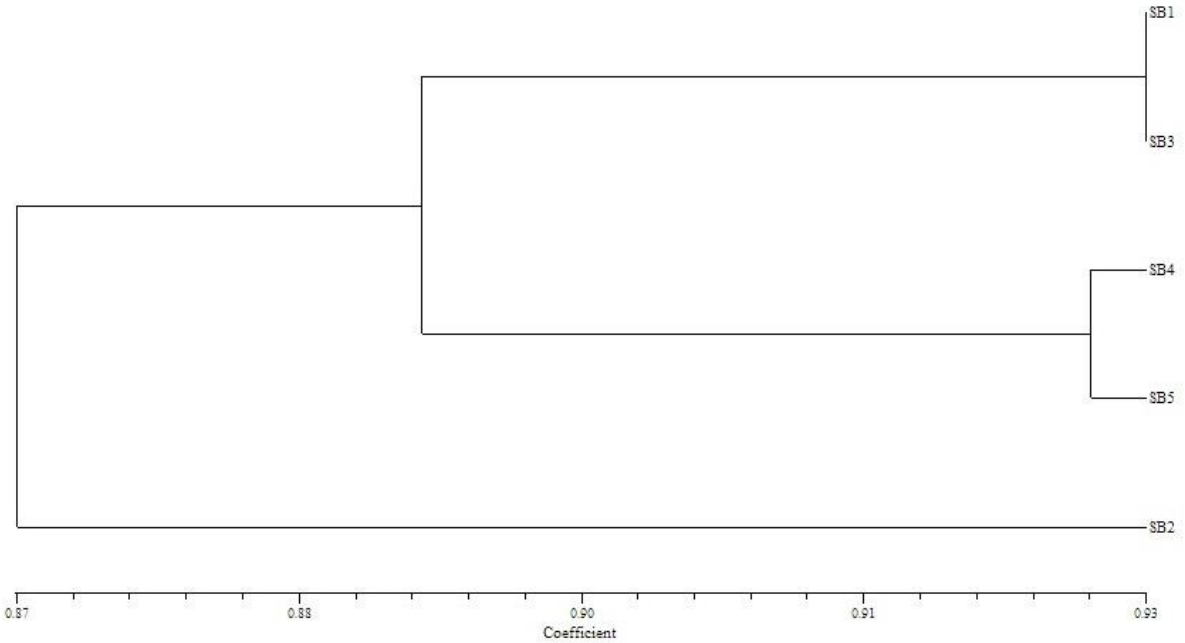

Figure 1. Dendrogram showing genetic relationship of five genotypes of *Tinospora cordifolia* (Willd.) Miers.

In the present study, Jaccard's similarity coefficient was used to assess the genetic similarity among different samples of *Tinospora cordifolia* using NTSYS pc version 2.0. A dendrogram was constructed using the similarity matrix and UPGMA by taking into account the presence (1) and absence (0) of bands. Out of Five genotypes, four form a single cluster. Genotype SB2 form a separate cluster. The first cluster included two subclusters formed by SB1 and SB3. The second subcluster included the genotype SB4 and SB5 was obtained. The similarity indices ranging from 0.87–0.93. Maximum 0.93 similarity was exhibited by both the genotypes namely, SB1 and SB3 as depicted in the dendrogram (Fig. 1). Anand (2003) reported about 97% homology between the mother plants and micro-propagated plants in an endemic and endangered plant, Syzygium *travancorium.* From the above finding, negligible polymorphism was detected during the marker analysis of

micro-propagated plants of *Tinospora cordifolia*. No phenotypic changes were observed in the micro-propagated plants which suggests the changes in the basis of DNA present in the repetitive sequences of DNA (non-coding regions) which impose a negligible effect on gene expression (Guo *et al*. 2006). Samantaray & Naiti (2010) suggested the use of meristematic tissues for in vitro culture initiation which is essential to maintain the genetic stability in micropropagules. Meristematic tissues are more resistant to genetic variation that might occur during cell division (Shenoy & Vasil 1992). A low level of genetic variation is reported in which use of RAPD markers for assessing the genetic fidelity of micro-propagated plants has been reported by several workers (Hashmi *et al.* 1997, Mondal & Raddy 2006, Saifullah *et al*. 2011, Khatab & Youssef 2018) whereas some suggested that plants raised by using micropropagation should be true-to-type plantlets with no genetic or morphological alteration (Rahman & Rajora 2001, Sudipta *et al*. 2014, Prakash *et al*. 2016, Safarpour *et al*. 2017). It was proven that the genetic fidelity of regenerated plants was maintained indicating high genetic stability among the clones (Moharana *et al*. 2018). The small variations detected by RAPD markers could be due to minor genetic rearrangements made during the regeneration of tissue through micropropagation. On the other hand, *in vitro* stress may cause the genome to respond by DNA methylation, and this may modify the RAPD profile through the insertion or excision of transposons (Hirochika *et al.* 1996).

CONCLUSIONS

From the present study, it has been concluded that the RAPD-PCR is the best, simple and cost-effective marker for the confirmation of the genomic integrity of *in vitro* raised plants from *Tinospora cordifolia* motherstock. The present method used in this study might be useful to know the genomic background of the medicinal plants with high commercial value, for monitoring the genetic stability of *in vitro raised* germplasm collections and efficient and effective management of cryopreserved plant material.

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