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Studies on Genetic Stability of Micropropagated Plants and, Reintroduction in an Endemic and Endangered Taxon: *Syzygium travancoricum* Gamble (Myrtacae)

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Abstract

Tissue culture techniques arguably are an important approach for *ex situ* conservation of rare and endangered plant species. However, there is utmost importance on maintaining the genetic integrity of the introduced plants especially in tree species. To examine the genetic integrity of the micropropagated plants, we randomly screened few hardened plants of *Syzygium travancoricum*, a critically endangered tree taxon, using Randomly Amplified Polymorphic DNA (RAPD) markers. Twenty-three random primers were tried and twenty-five polymorphic loci were identified. The dendrogram based on the Unweighted Pair-Group Method Arithmetic Average and Nei's similarity index depicted about 97% homology between the mother plants and micropropagated plants. Further, an attempt was made to reintroduce the micropropagated plants in the wild. Over three hundred small trees could be successfully established.

Key words: Conservation, Endangered plant, Genetic stability, Micropropagation, RAPD, Reintroduction

Abbreviations: RAPD- Random Amplified Polymorphic DNA; UPGMA- Unweighted Pair Group Method with Arithmetic averages

Introduction

Conventional techniques supplemented with *in vitro* manipulation of plus trees (higher levels of diversity, growth vigour) provide an excellent means for conserving rare and endangered plants (Bajaj 1986; Vasil and Vasil 1990; Malda et al. 1999). *In vitro* culture is an efficient method for *ex situ* conservation of plant diversity (Fay 1992; Krogstrup et al. 1992; Fay 1994) since this provides a useful tool for mass multiplication and their reintroduction, with minimum impact on wild populations. Recently, few reports have demonstrated the versatility of *in vitro* techniques for multiplication and reintroduction of rare and endangered plants of the Southern Western Ghats of India (Anand and Rao 2000; Anand et al. 1999; Seeni and Latha 2000; Jayanthi and Mandal 2001).

Syzygium travancoricum Gamble is a critically endangered and endemic medicinal plant distributed along the swampy marshes in the sholas of Southern Western Ghats, India (Nayar and Sastry 1987). They are small trees (8-10m) occurring at an altitude of 700-1500 m above sea level. The bark and the berries of the plant are used in local medicines (Anonymous 1956). The plant is a critically endangered species on the basis of population reduction and the population estimate of less than 200 matured trees in the wild (IUCN 2000). Owing to its critical state in wild, there is an urgent need to conserve this taxon. Keeping this in mind an efficient tissue culture protocol for the mass multiplication was standardised in this taxon, through rapid axillary bud proliferation and indirect organogenesis (Anand et al. 1999). Apart from developing protocols for mass multiplication, viable *in vitro* cultures were maintained on minimal medium supplemented with osmoticum (mannitol, sorbitol) without sub-culturing for over 8 months (Anand 1999), enabling the possibility of long-term preservation of the germplasm.

Tissue culture often has the limitation of introducing genetic variability in the propagated species that can be monitored by using several molecular marker systems from time to time. The DNA-based markers are preferred over others due to the inertness of the DNA to developmental, physiological or environ-

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mental changes for screening the variation induced under *in vitro* condition. Random Amplified Polymorphic DNA (RAPD) analysis (Williams et al. 1990; Williams et al. 1993) using oligonucleotide primers of variable lengths (8-12 oligomers) was demonstrated to be useful marker in characterizing the genetic fidelity of the tissue culture plants (Parani et al. 1997; Rani and Raina 1998; Rani and Raina 2000; Jayanthi and Mandal 2001). This technique has advantage over others in the availability of unlimited number of markers and no prior information of the genome is required as is the case of many wild species. In the last decade, various molecular marker systems have been developed and used although the use of RAPDs still seems to be most common, despite the weakness of this marker system (Karp et al. 1998; Rao and Hodgkin 2002).

In vitro manipulation through micropropagation and rehabilitation of the endangered plants into its original or favourable habitats is one of the strategies for conserving a species in complementation with other *in situ* conservation practices. Screening the tissue culture-derived plants at an early stage or during hardening and prior to re-introduction using molecular-markers will assist in reintroducing true-to-type plants (Heinze and Schimidt 1995), and protecting their genetic integrity. This study was aimed to validate the genetic integrity of micropropagated plants derived through axillary bud proliferation using RAPD markers. We also report our limited success story with reintroduction of the micropropagated plants of this endangered taxon.

Materials and Methods

Plant materials and explants source

The plant material, media composition and culture conditions for *in vitro* multiplication using axillary bud meristems has been described previously by Anand et al. (1999). The *in vitro* rooted plants were washed free of phytagel and hardened in growth chambers (NK, systems, Japan) under high humidity (Anand et al. 1999). Once established under growth chamber conditions, the plants were moved to green houses (30±4°C, 75% RH and 14:10h photoperiod) for further acclimatization. Fifteen micropropagated plants from the above stage of hardening were selected randomly for screening their genetic integrity. All these plants were derived from a single mother plant (the source for explants), which was also included in these analyses. Young *in vitro* regenerated plantlets approximately 8-10 cm in height and young leaf tissues from the hardened plants in the greenhouse were used for DNA extraction.

Extraction of genomic DNA and Random Amplification Polymorphism DNA (RAPD) Analysis

About 200 mg fresh weights of the young tissues were used for DNA extraction. Genomic DNA was extracted according to protocol described by Rogers and Bendich (1988) with minor modification. Two independent DNA preparations from the mother plant (the source of explants) were tested with 10 primer/DNA combinations for reproducibility of the RAPD fingerprints. Polymerase chain reaction (PCR) for RAPD fingerprinting, was carried out using 23 arbitrary 10-mer oligonucleotide primers (Operon Tech, USA) / DNA combinations. Each reaction mixture of 25 μL contained 10 ng of DNA, 2.5 μL of assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 0.25 mM dNTP, 0.2 μM of oligonucleotide primer and 0.5 U Taq DNA polymerase (Bangalore Genei, India). The reaction mixture was overlaid with equal volume of mineral oil, and amplification was performed in 0.2 mL microfuge (Dialabs). DNA amplification were carried out in MJ Research, Mini Thermal cycler (USA) for 30 cycles using an initial denaturation at 94°C for 4 min, followed by 30 cycles of 1.0 min at 94°C, 1.5 min at 38°C and 2.0 min at 72°C. One additional complete extension cycle was performed for 10 min at 72°C. The amplified fragments were analyzed on 1.4% (w/v) agarose gels (SRL, India) using 2.5μL of tracking dye (Bromophenol blue + xylenecynol) and stained with an ethidium bromide (10 mg/mL) solution. The gels were photographed under UV in a transluminator and documented using the gel doc system (Pharmacia, India).

Statistical Analysis

All reactions were performed thrice and only those bands that were reproducible were scored for presence/absence (1/0) of bands. Percentage polymorphism was calculated as the proportion of amplification products, which were polymorphic across all the lanes to the total number of amplification products. Pairwise comparisons of RAPD markers from individual plants were made to calculate Nei's similarity indices (Nei 1972). Cluster analysis was carried out using unweighted pair group (UPGMA) with arithmetic mean method (Sneath and Sokai 1973).

Reintroduction

The rooted plants, removed from the flasks were washed free of phytagel, transferred to polythene bags containing sterilized sand and soil mixture (1:1) and moved into growth chambers (NK Systems, Japan) at 70% humidity and 28±2°C for hardening. The plants were fertilized with $1/8^{th}$ MS macronutri-

ents twice during the course of hardening (approximately 4-6 weeks) at an interval of two weeks. The well-established plants in the growth chambers were further hardened in the greenhouses (30±4°C, 75% RH and 14:10 h photoperiod) for a period of 2 months, before being transported on trucks to the field sites (approximately 400 km from the micropropagation facility based in Madras). The reintroduction program was carried out in two different sites; The gene pool reserve of Gudalur with the involvement of the forest department, and in the Wynad forests with the help of local people and our staff based in this field station. In the first phase of reintroduction, 800 micropropagated were introduced in the gene pool reserve (native and semi-native habitats). Planting was carried out before the onset of the northeast monsoon (October-December). Provisions were also made for watering the plants in the late summer. The mortality rates were recorded introduction and subsequently in the following years. In the second phase, 1500 micropropagated plants were reintroduced of which 125 plants were introduced in Wynad (including the 15 plants selected for RAPD screening), while 1250 of the micropropagated plants were introduced in the gene pool reserve, Gudalur. The establishment rate and the mortality rate were recorded.

Thirty-five healthy looking plants (including 5 well established plants selected for RAPD screening) each were randomly tagged from Gudalur and Wynad sites. The growth performances of these plants were recorded for the following parameters *viz*: Height of the plant, internodal length, number of lateral roots, number of new offshoots, leaf length and leaf breadth. The data were statistically analyzed and growth performances were recorded with mean and standard deviation for a period of two years.

Results

RAPD analysis

The DNA extraction from the young plants was a problem due to the high amounts of phenolics in the juvenile tissues often leading to browning and poor quality of DNA. A slight modification in the DNA extraction protocol involving re-extracting the DNA emulsion with 1/10 volume of 10% CTAB (10% CTAB; 0.7 M NaCl) and an equal volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0) overcame the problem of phenolics and facilitated the recovery of good quality of DNA for Polymerase Chain Reaction (PCR). The good quality DNA recovered was initially tested by PCR using few random primers for the reproducibility of amplified products. For reconfirming the reproducibility of the RAPD fingerprints for DNA/primer combinations, two independent DNA extracts prepared from the mother plant was tested for a minimum of 10 primers. Similar banding patterns for the independent DNA extracts were observed for all the DNA/primer combinations tested (Figure 1). DNA from these two independent preparations of the mother plant was pooled together for future experiments.

A total of 23 primers were employed in the present study, and 17 of them gave reproducible results under similar conditions, which were scored for assembling a matrix of genotypes. A total of 78 bands were amplified and among them 25 were polymorphic. The number of amplification products and the number of polymorphic bands detected for each primer/DNA combination is depicted in Table 1. The number of amplification products per primer ranged from two (OPB02, OPB04) to eight (OPAB09). Averages of 4.6 RAPD markers were amplified per primer. The number of polymorphic bands per primer ranged from zero (OPB02, OPB03, OPB04, OPB11, OPAB04, OPAB07, OPAB11) to five (OPB08). The RAPD fingerprints of 15 micropropagated plants and the mother plant for primers OPAB08, OPAB09 and OPB05 is shown in Figure 2.

A total of 1056 bands were scored and 81 bands were found polymorphic (a frequency of 7.6% polymorphism). The dendrogram based on 78 RAPDs showed fourteen micropropagated plants and the mother plant formed a single large cluster at an estimated genetic distance of 0.08 (Figure 2) {97% similarity}. Six micropropagated plants (b, f, g, i, k and l) showed highest degree of similarity with the mother plant, while two plants (e and o) were the least similar. The cluster diagrams of the genotypes are shown in Figure 3.

Reintroduction of the micropropagated plants

The micropropagated plants were grown in the forest nurseries for a period of 6-8 months before moving to the gene pool

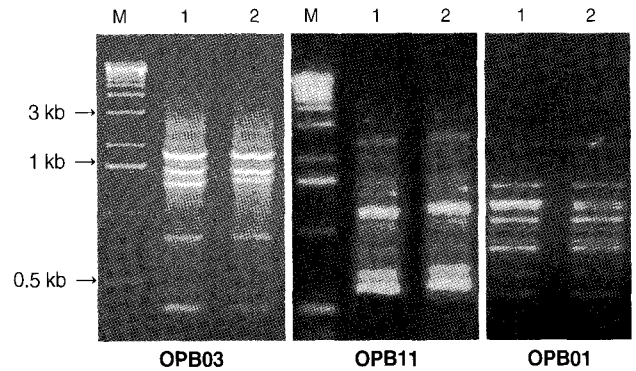

Figure 1. The RAPD fingerprints generated from two independent DNA extracts of the mother (donor) plant are identical. Lanes: M: Molecular size marker; and 1-2: Two independent DNA extracts from the donor mother plant. Details of the primers are provided with the figure and arrows indicate the expected molecular sizes on 1-kb ladder.

reserve area (Figure 4A). In the first phase, seventy five of the tissue culture plants were established. Total survival rate of about 10% was achieved by the end of one-year period in the first phase of reintroduction. In the second phase of reintroduction, the percentage establishment rate in both the Wynad and Gudalur sites ranged between 44% and 14% respectively. A total of 58 micropropagated plants that included 8 out of the 15 plants selected for genetic fidelity studies were established in Wynad. Eight of the micropropagated plants that was selected for the genetic fidelity studies and reintroduced in the gardens of the local tribes (including all the 8 surviving plants) showed great establishment success (Figure 4B), however the remaining seven scored for genetic fidelity and reintroduced in wild did not survive. In Gudalur, only 168 plants from the pool of 1250 plants reintroduced survived and overall 301 micropropagated plants (13.2%) that survived first year in wild were well established by the end of this investigation. These plants were about 2-3-year old small trees.

The growth performance based on the 2nd year field data indi-

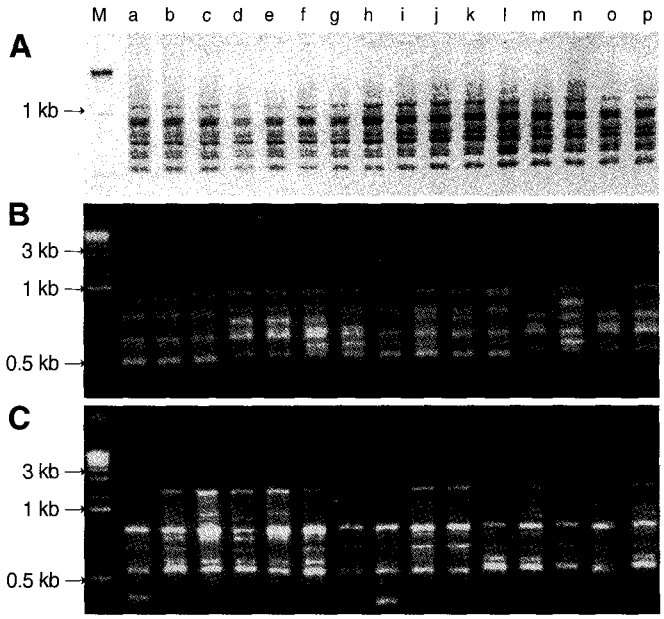

Figure 2. Amplification products generated from fifteen tissue culture-derived and the mother plant of *Syzygium travancoricum* using RAPD primers; A) OPAB09; B) OPB08 and; C) OPAB08. Lanes: M: Molecular size marker; a: Donor mother plant; b-f: Plants derived from the axillary proliferation stage; g-k: Plants regenerated from the nodular calli; l-p: Plants from the hardening stage. Arrows indicate the expected molecular sizes on the 1-kb ladder.

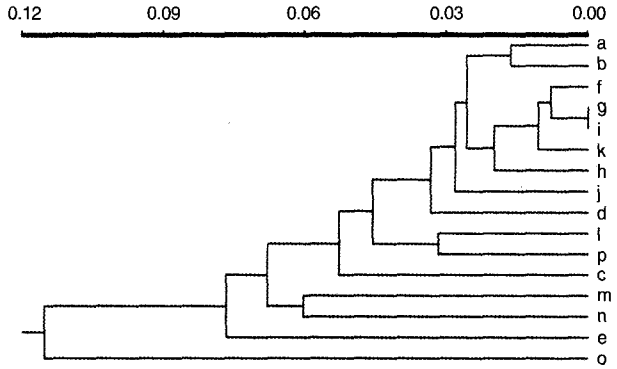

Figure 3. Dendrogram of fifteen hardened plants and the mother plant of *Syzygium trvancoricum* generated by UPGMA cluster analysis, based upon 78 RAPD depicting the genetic similarity between the individual plants. Fourteen of the *in vitro* plants formed a single cluster with the mother (a), while plant 'o' fell apart from this clustering.

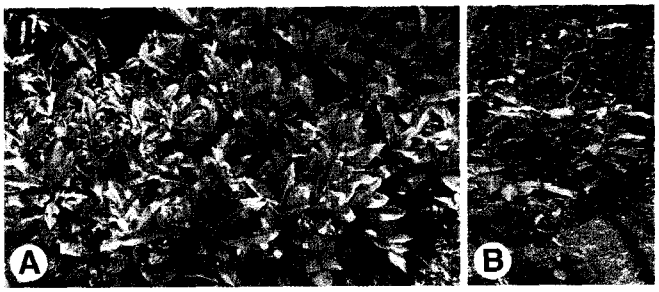

Figure 4. Reintroduction of the micropropagated plants in the wild. Panel A; Hardenig of the micropropagated plants in the forest nurseries. Panel B; A reintroduced tissue culture plant of *Syzygium travancoricum* established and maintained by a local tribe in Wynad. This plant is about 3-yrs old.

Table 1. The names and sequences of the 17-oligonucleotide primers used for generating 78 RAPD markers for analyzing the genetic fidelity of the tissue culture-derived plants at different stages of their propagation.

Primer	Total no. of bands	No. of polymorphic bands
OPB01	6	3
OPB02	2	0
OPB03	4	0
OPB04	2	0
OPB05	3	1
OPB06	5	2
OPB08	7	5
OPB11	6	0
OPB13	4	1
OPB14	5	1
OPAB01	4	3
OPAB03	4	2
OPAB04	3	0
OPAB07	3	0
OPAB08	8	4
OPAB09	8	0
OPAB10	4	3
Total	78	25

cated that the plants transferred in Wynad (including five of the micropropagated plants selected for genetic stability studies; Individual data not shown) grew more vigorously (Table 2). The average number of new shoots including the lateral shoots ranged anywhere between (14.4 and 17.1 shoots) with larger internodal distances (10 cm) in the plants introduced in Wynad. These plants were much taller (2 m) with larger leaves (20.5/9.2 cm) when compared to those from Gudalur (Table 2). Micropropagated plants reintroduced in Wynad performed better for all the growth parameters analyzed including lower mortality, better growth and better establishment rates.

Discussion

The visual assessment of over hundred plus plants derived from the axillary bud culture did not rev al any morphological variation in the micropropagated plants. The inertness of the well differentiated apical or bud meristems to a limited or no variations under tissue culture conditions are very well documented (Vasil and Vasil 1990; Wang and Charles 1991; Valles et al. 1993; Rani and Raina 1998, Jayanthi and Mandal 2001). The identification of variability in micropropagated plants derived from the same donor mother plant as in *Populus deltoides* (Rani et al. 1995) and *Piper longum* (Parani et al. 1997) using RAPD and in few other cases however provides evidences for the existence of variants. Thus suggesting visual phenotypic evaluation may not be sufficient for characterizing the *in vitro* plants. Screening DNA variations among several millions of base-pair could be more problematic and exhaustive than scoring for a few morphological variations. There are several reports documenting similar observations as for somoclonal phenotypic variants in begonia (Bouman and De Klerk 2001); Tomato (Smulders et al. 1995) and in *Picea* somatic embryo-derived plants despite major chromosomal aberrations (Fourre et al. 1997).

The present investigation using RAPD markers detected variation that were not confined to few individuals but distributed among the micropropagated plants. The dendrogram based on UPGMA showed that fourteen of the regenerated plants and the mother plant formed a single large cluster and were 97% genetically identical to the mother plant. Similarly, the mother plant (a) and micropropagated plants clustered into fourteen separate groups for the limited number of markers employed. The observed small variations in DNA may have occurred *de novo* during the period of dedifferentiated cell proliferation between the culturing of the explant and tissue regeneration. This data is more or less in accordance to earlier observations on minimal variability in micropropagated plants of *Populus deltoides* (Rani et al. 1995), *Panax notoginseng* (Shoyana et al. 1997) and *Tylophora indica* (Jayanthi and Mandal 2001) using RAPD markers. Incidental changes in clonally propagated plants have been previously described by many authors that may arise from the misinterpretation of presence/absence of bands and as artifacts of the method that are not true differences (Smulders et al. 1995; Fourre et al. 1997; Jayanthi and Mandal 2001). Molecular markers like RAPD can cover only very small portions of the genome even when 100's of primer are employed (Bouman and De Klerk 2001) and since few of the micropropagated plants selected for RAPD screening did not survive in the wild after reintroduction, the interpretation of the true-to-typeness of the regenerants needs further justification. We therefore propose to extensively screen the surviving 8 micropropagated plants (scored for genetic stability) for morphological and genetic variations and to further increase the number of plants screened for genetic fidelity in future to validate these findings.

Although many workers have suggested the role of *in vitro* propagation of rare/and endangered plants as an effective means for conservation (Bajaj 1986; Vasil and Vasil 1990; Malda et al. 1999) there are limited reports on reintroduction of micropropagated plants in wild (Seeni and Latha 2000) which are limited to *ex situ* conservatories of many Botanical gardens (Royal Botanic Gardens, Kew, UK; Mount Annan Botanic Garden, Australia; Cincinnati Zoo and Botanical Gardens, Ohio, USA etc.). About 2300 micropropagated plants were successfully acclimatized and hardened under field conditions prior to their reintroduction, which is in accordance to our earlier reports of 40% and above survivability rate in the hardened plants (Anand et al. 1999). The mortality rate in the reintroduced plants was very high during the first year of reintroduction; This must have been compounded due to the extremes of the environmental pressure and poor reestablishment status of the taxon.

The field data recorded for six different parameters (data for 2nd year recorded in Table 2) indicated that the plants in Gudalur did not perform as well as those in Wynad. This could

Table 2. Growth performance of the reintroduced micropropagated plants from two different sites: Gudalur and Wynad. Data recorded is the mean of thirty-five healthy looking plants tagged from each site after two years of growth.

Growth parameters	Sites	
	Wynad*	Gudalur*
Height (m)	2.03±0.14	1.47±0.21
Internodal length (cm)	10.1±1.66	7.07±0.73
Number of lateral shoots	14.4±3.31	6.4±1.83
Number of new shoots	17.15±4.03	4.9±2.61
Leaf length (cm)	20.44±1.87	18.43±2.24
Leaf breadth (cm)	9.27±0.96	8.65±1.11

* Mean±SD

be attributed to the difference in the demographical positioning of the two sites, the greater environmental stress (longer dry spell) and the poor soil nutrient table. The awareness program in Wynad by the staff of the foundation led to better understanding of the ethnic perception of this taxon and the general acceptance of the critical state of the plant in wild. This facilitated in attracting local tribes to collect and plant the micropropagated plants in their gardens (Figure 4B). The plant in the wild is limited by its population (IUCN 2000; Anand 1999), which is less than few hundreds, thus making our limited success with reintroduction itself a significant achievement in saving the species. Burgess (1994) stressed that understanding the ethnic perceptions of any taxon is critical to the *in situ* and *ex situ* conservation and enhancing the effectiveness for educational efforts for conservation. In conclusion, the plant species requires immediate attention with greater emphasis for habitat protection and incorporation of larger germplasm collection and other propagation techniques.

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