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ORIGINAL ARTICLE

Comparative assessment of variation in the USA *Arachis pintoi* (Krap. and Greg.) germplasm collection using RAPD profiling and tissue culture regeneration ability

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Abstract *Arachis pintoi* accessions were used to study genetic diversity using RAPD markers. Concurrently, two tissue culture protocols were evaluated for organogenesis and the capacity to generate somaclonal variation. Data were collected on callus growth, callus weight gain, and number of regenerated plants. Robust RAPD profiles were obtained and eight primers amplified 100 different bands with 98% polymorphisms. The proportion of polymorphic RAPD loci was 89%. Average genetic distance was 0.36 and indicated that a large amount of genetic diversity exists within the germplasm evaluated. Genetic distances were used to prepare a dendogram for the *A. pintoi* accessions that separated them into four groups. A large degree of variability for callus induction and callus weight gain was observed among the accessions. Shoot regeneration was achieved for several accessions on both media with no structures indicative of somatic embryogenesis detected. Root induction was difficult to obtain, and many shoots died during this process. RAPD band profiles of regenerated tissue culture plants were similar to their parent plants, and therefore no somaclonal variation was evident using these methods.

Keywords Genetic resources · RAPD marker · Tissue culture · Tropical legume

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Introduction

Arachis pintoi Krap. and Greg. is a herbaceous, perennial legume, exclusively native to Brazil. It is considered a multiple use legume because it is grown for forage, as ground cover in orchards, forests, and low tillage systems, for erosion control, and for ornamental purposes. As a result of the worldwide distribution of a single accession (PI 338447) by the international germplasm exchange network, which is led by Bioversity International and the national germplasm centers, several cultivars have been released in nine countries, including Australia, Brazil, Colombia, Costa Rica, Honduras, and the USA (Perez and Pizzarro 2005; Argel and Villarroel 1998; Pereira et al. 2004).

Until the early 1990s, *A. pintoi* had a narrow genetic base. However, the importance of the genus *Arachis* and the success of the forage cultivars developed led to an intense germplasm collection of the species. Consequently, the germplasm base grew to more than 150 accessions (Valls and Pizarro 1994). Today, this large number of accessions has resulted in the need for accurate methods to discriminate among them for more effective use as breeding materials. Characterization and evaluation of these available resources will allow the establishment of core collections (core collections) that, by definition, embrace the maximum genetic diversity contained in cultivated species and in the related wild species, with the minimum possible number of accessions (Frankel and Brown 1984).

Germplasm characterization generally consists of studies of eco-geographic and demographic adaptation (Martins 1984), and according to Solbrig (1980) involves mostly the parameters of the vital cycle of the organism, genetic and physiological studies, plant pathology, and yield evaluation, among other studies. Often, characterization also involves taxonomic confirmation and should produce an easy and quick way to differentiate the germplasm using highly heritable and visible traits (Hawkes et al. 2000). Ideally, breeding programs should begin only after appropriate germplasm characterization (Cameron 1983).

Among the DNA-based markers, random amplified polymorphic DNA (RAPD) has been the most popular with plant breeders. In a recent bibliographic search, there were more than 2,000 publications in which RAPD markers had been used (Reiter 2001). According to the same study, the popularity of this marker is due to the fact that the method is relatively simple and low cost. RAPDs have also been used as markers of choice in several genetic studies with cultivated peanut (*Arachis hypogaea* L.) and its wild relatives (Hilu and Stalker 1995; Chang et al. 1999; Nóbile et al. 2004).

Tissue culture techniques also play an important role in genetic resources research. Potential applications of tissue culture are: micropropagation, long-term storage, germplasm exchange, embryo culture of interspecific hybrids, induction of mutations, production of synthetic seed, and genetic transformation. Although in vitro techniques are useful for genetic resources conservation, attention must be given to the genetic stability of the systems used in the process. Genetic variation has been observed among regenerants of several species, and they usually present mutations that include: chromosome breakage, changes in ploidy number, single base changes, changes in copy numbers of repeated sequences, increased transposon mutagenesis, sister chromatid exchange, and alteration in DNA methylation (Hawkes et al. 2000).

Because in vitro techniques have a wide spectrum of applications for genetic resource conservation and plant breeding, the development of protocols optimized for a particular species should have high priority. Several factors may affect efficiency of the process and should be considered when research is conducted in this field. Genotype, explant source and age, hormones and their concentrations in the medium, and day length are several factors that affect the success of regeneration of plants via tissue culture (Flick et al. 1983).

Tissue culture regeneration protocols for *A. pintoi* have been developed based on a single genotype (Rey et al. 2000), or based on a small number of accessions (Ngo and Quesenberry 2000). In terms of preservation of genetic resources, it is critical to evaluate the efficiency of tissue culture protocols for their ability to regenerate plants in a genotype-independent manner while also preserving the genetic characteristics of the original, parental germplasm. Additionally, in terms of plant breeding, it is important to assess the genetic diversity of the germplasm with respect to their plant regeneration abilities in vitro and to estimate the capacity of given tissue culture protocols to generate somaclonal variation, which ultimately could generate mutations and increase gene diversity.

The objectives of this study were threefold: to characterize the *Arachis pintoi* germplasm accessions stored at the USDA-NPGS germplasm banks using RAPD markers, to evaluate plant regeneration of these *A. pintoi* accessions via organogenesis using two different tissue culture protocols, and lastly to study the degree of variation indicated by RAPD profiles of tissue culture-derived plants compared to their parent plants.

Materials and methods

Thirty-four *A. pintoi* accessions (Table 1) stored at the National Plant Germplasm System (NPGS/USDA, Griffin, GA) were used to study the genetic diversity of this collection at the molecular level using RAPD markers. In addition to the RAPD characterization of the "parent plants," 25 of the 34 accessions were selected randomly and evaluated for their ability to regenerate by organogenesis using two tissue culture protocols. The extent of somaclonal variation was accessed by comparing the RAPD profiles of the regenerated tissue culture plants to their parent plants.

Genomic DNA extraction

DNA was extracted from single plants, stored at the Agronomy Department greenhouse (University of Florida, Gainesville, FL), originated from rooted vegetative cuttings received from the National Plant Germplasm System (NPGS/USDA, Griffin, GA). Stems of *A. pintoi* germplasm accessions were placed in vermiculate trays under an automated mist system (10 s every 30 min) for rooting. After 45 days under the mist systems, the rooted cuttings were transferred to 150 cm^3 Conetainers® filled with methyl-bromide-fumigated fine sand topsoil. Leaf tissue was collected and placed in 50-ml centrifuge tubes (Corning Incorportaed, Corning, NY) and stored in a container with liquid nitrogen, and then in a −70°C freezer.

Genomic DNA was extracted from 0.1 g of ground young leaf tissue of single plants using a modified CTAB protocol (Rogers and Bendich 1985). DNA was washed with 80% ethanol, dried, and resuspended in 50 µl of DDW. Working solutions were prepared by diluting the

Table 1 *Arachis pintoi* accessions charaterized by RAPD profiling and tissue culture regeneration ability

PI/CIAT number	Lat. (south)	Long. (west)	Altitude (m)
476132	16°08′	47°12′	690
497541	18°38′	44°04′	640
497574	13°23′	44°05′	450
604798	16°18′	46°58′	630
604799	16°19′	46°51′	580
604800	16°41′	46°29′	540
604801	16°42′	46°25′	560
604803	14°25′	44°22′	510
604804	14°20′	44°25′	560
604805	16°59′	45°57′	570
604807	13°18′	46°48′	510
604808	13°18′	46°42′	500
604809	13°02′	46° 45′	610
604810	13°06′	46°45′	600
604811	13°51′	46°52′	490
604812	14°28′	46°29′	500
604813	14°27′	47°00′	480
604814	15°52′	39°08′	50
604815	15°49′	47°58′	1,080
604817	18°38′	44°04′	630
604856	16°53′	42°07′	360
604857	13°23′	44°05′	450
604858	15°26′	47°21′	700
604859	17°03′	42°21′	360
18745	16°05′	42°05′	280
20826	22°55′	47°05′	–
22150	15°07′	44°08′	510
22152	16°52′	46°5′	550
22159	15°17′	47°23′	650
22234	13°14′	46°44′	463
22256	16°10′	46°01′	580
22260	14°04′	47°18′	720
22265	16°04′	44°01′	–
22271	15°47′	47°56′	1,040

DNA stock solutions with DDW and standardized to concentrations of 25 ng of DNA per µl.

RAPD profiling

Genomic DNA of each tested accession was screened with 18 primers [A04, A10, A12, A15, B04, B05, B10, B16, C02, C04, D04, D13, E04, E05, E08, E15, G05 and G15 (Operon, Alameda, CA] using RAPD analysis of the PCR products for each accession.

Amplifications were performed in a PTC-100 DNA thermocycler (MJ Research, Watertown, MA) under the conditions described in Gimenes et al. (2000). Amplification products were separated by electrophoresis on 1.5% agarose gels, and a 1-kb DNA ladder (Promega Coorporation) was used as a molecular size marker. Banding profiles were visualized by staining the gels with ethidium bromide (10 mg/ml) and viewing under UV radiation. Images were captured with Quantity One quantification software (version 4.1.1, BIO-RAD Laboratories, Hercules, CA), and then bands were scored. For each combination of accession and primer, five PCR reactions were performed. Only reproducible bands occurring in at least three out of five gels were evaluated. These RAPD bands were scored as present (1) or absent (0) for all accessions, and a phenotypic binary matrix was produced. This matrix was used to perform genetic analysis using the software POPGENE (version 1.32; http://www.ualberta.ca/~fyeh/index.htm). Allele frequency, number of polymorphic loci, Nei's genetic distance (Nei 1972), Nei's genetic diversity index (Nei 1973), and Shannon-Weaver's genetic diversity index (Shannon and Weaver 1949) were the parameters calculated.

Nei's gene diversity is defined as $h = 1-\Sigma x_i^2$, where x_i is the frequency of the ith allele. Additionally, Shannon-Wiever's diversity index is defined as: $H = -\Sigma$ ((pi log pi)/log pi), where $I = 1$ to n, and p is the proportion of the total genotypes made up to the ith genotype. In both indices, values close to one indicate high genetic diversity.

According to Nei (1972), genetic distance is calculated using the following formula: $D = -\log_e I$, where $I = Jxy/(JxJy)^{1/2}$, Jxy is the number of bands in common among accessions x and y, and Jx and Jy are the number of bands of accessions x and y, respectively. I values equal 1 when two accessions or populations have identical gene frequencies over all loci examined, and zero when they share no alleles. Genetic distance ($D = -\ln I$) was later used as a criterion for differentiation among accessions to prepare a phenogram constructed from the calculated distance relations, using the unweighted pair group method (UPGMA).

Tissue culture

Two tissue culture protocols, Protocol 1 developed by Rey et al. (2000) and Protocol 2 used by Ngo and Quesenberry (2000), were used for each accession, and data were collected on callus growth, callus weight gain, and the number of regenerated plants. The experiment was conducted using a completely randomized design with 50 treatments and three replications. Callus growth was rated on a 1–5 scale, where 1 = no callus growth and 5 = the largest amount of callus growth. In addition, callus weight was measured 28 days after incubation, at the moment of the transferring to the shoot induction medium.

Leaflet pieces from adult plants were the explant sources for callus initiation. Leaflets were surface sterilized by

immersion in 70% ethanol for 1–2 min, followed by immersion in a solution of commercial bleach (0.9% sodium hypochlorite, final concentration) plus one drop of Tween® for 1–2 min. Leaflets were then washed three times with autoclaved distilled water. Circles of approximately 19.4 mm^2 area of the median portion of the laminae were cut and placed with the abaxial side down on the media in 60 mm × 15 mm petri dishes.

Protocol 1

The callus induction medium consisted of major and minor salts, as well as vitamins according to Murashige and Skoog (1962), with 3% sucrose, 0.7% agar, and 10 mg l^{-1} NAA (1-naphthaleneacetic acid) +1 mg l^{-1} BA (6-benzylaminopurine). The pH of the medium was adjusted to 5.7 with 0.1 N KOH or HCl prior the addition of agar. To induce shoots, small pieces of callus (30 mg fresh weight) were transferred to fresh medium composed of MS basal salts and vitamins +1 g l^{-1} BA. For root induction, the regenerated shoots were transferred to a medium composed of MS basal salts and vitamins +0.01 mg l^{-1} NAA (Table 2).

Plantlets resulting from rooted shoots were rinsed gently under running tap water to remove adhering cultured medium and immediately planted in pots containing commercial potting mix (Miracle-Gro® Moisture Control® Potting Mix, Scotts Miracle-Gro Company, Marysville, OH). These plants were acclimatized in a humidity box and then placed in the greenhouse.

Protocol 2

The MS basal salts and vitamins medium (Murashige and Skoog 1962) was the base for all media used in this protocol. For callus induction, the MQC medium, which is a modification of the MS medium proposed by Wofford et al. (1992) for tropical legumes, was the medium of choice. In this medium, a 2:1 auxin/cytokinin ratio was employed, with a final concentration of 2 mg l^{-1} of IAA (indole-3-acetic acid) and 1 mg l^{-1} of kinetin. The pH was adjusted to 5.8 prior to autoclaving.

For shoot development, the medium composed of MS basal salts and vitamins plus 0.1 mg l^{-1} of IBA (3-Indol butyric acid) was employed. For root induction, the regenerated shoots were transferred to medium containing MS basal salts and vitamins with growth regulators (Table 2).

Plantlets resulting from rooted shoots were rinsed gently under running tap water to remove adhering cultured media and immediately planted in pots containing potting mix. These plants were acclimatized and then placed in the greenhouse.

DNA was extracted from regenerated plants in accordance with the protocol described earlier. PCR reactions, band separation, and scoring were also identical to that used for the parent plants. RAPD profiles of the regenerated tissue culture plants were compared to the parent plants.

Table 2 Tissue culture protocols used to regenerate *Arachis pintoi* plants

Protocol	Callus induction medium	Shoot development medium	Root induction medium
1	MS[a]	MS + 1 g l^{-1} BA	MS + 0.01 mg l^{-1} NAA
2	MQC[b]	MS + 0.1 mg l^{-1} IBA	MSNH[c]

[a] MS + 3% sucrose, 0.7% agar, and 10 mg l^{-1} NAA + 1 mg l^{-1} BA

[b] MS + 3% sucrose, 0.7% agar, and 2 mg l^{-1} of IAA and 1 mg l^{-1} of kinetin

[c] MS basal salts and vitamins and no hormones

Results

Genetic analysis

Of the 18 primers tested, amplifications were obtained with eight of them. Primers A4, B4, B5, C2, D4, D13, E4, and G5 amplified 100 different bands. The average number of amplified bands per primer was 12.5. The size of these 100 fragments ranged from 250 to 3,500 bp. Of the 100 bands amplified, 98 were polymorphic. The average presence of bands per accession was 32, ranging from 20 to 44 bands. Ten bands were unique to an individual germplasm accession. Accession PI 604856 was discriminated by band C2-465 bp, accession PI 604858 by band E4-600 bp, accession PI 604810 by band C2-520 bp, accession PI 604799 band B5-1,250 bp, PI 604809 by band B5-1,100 bp, accession PI 604807 by band G5-1,750 bp, accession CIAT 22256 by D4-1,200 bp, accession CIAT 22159 by band D13-3,310 bp, CIAT 22152 by band B4-700 bp, accession CIAT 22265 by band D13-2,800 bp, and finally accession CIAT 22260 by band B5-500 bp. A summary of the results obtained with RAPDs among the 34 germplasm accessions of *A. pintoi* analyzed in this research is presented in Table 3.

Values of *h* (Nei's gene diversity) and *H* (Shannon-Wiever's diversity index) among the 100 RAPD loci measured were extremely variable with some loci having numbers close to one and others with numbers close to zero. In general, *H* values were higher than *h* values. To estimate the genetic diversity of the entire germplasm set,

Table 3 Characteristics of RAPD profiles of the 34 *Arachis pintoi* germplasm accessions

Total number of screened primers	18
Number of polymorphic primers	08
Total number of bands amplified	100
Size of the amplified bands	250–3,500 bp
Minimum and maximum number of bands per primer	08 (D4–16 (D13)
Average bands per primer	12.5
Total number of polymorphic bands	98
Total number of monomorphic bands	02
Average number of bands per accession	32
Minimum and maximum number of bands per accession	20–44
Number of accession-specific bands	10

the average of both indices was calculated and labeled as total genetic diversity. Total h was estimated at 0.29 ± 0.16, and total H was estimated at 0.45 ± 0.20. Both values are considered high, which demonstrates the great genetic variability contained in this set of germplasm.

Genetic distances (D) were calculated between every pair of accessions, and then the average genetic distance was estimated as 0.36. This value also indicates that a large amount of genetic diversity exits among the germplasm evaluated in this research.

Genetic distances were used to prepare a dendogram for the 34 *A. pintoi* accessions. Four groups were formed (Fig. 1). Group 1 contained accessions PI 604798, 604801, 604804, 604805, 604808, 604809, 604814, 604815, 604817, 604856, and 604857. Group 2 contained accessions PI 497541, 604800, 604812, 604858, 604859, and CIAT 18745, 20826, 22150, 22152, 22256, 22260, and 22265. Group 3 contained accessions PI 476132, 497574, 604803, 604807, 604810, 604811, 604813, and CIAT 22159, 22234, 22271. Finally, accession PI 604799 was the only member in group 4.

Organogenesis and plant regeneration

Callus induction was achieved using two different M.S. basal protocols after 28 days of culture. Analysis of variance demonstrated that Protocol 1 was superior to Protocol 2 for both variables related with callus growth. A large degree of variability for these two variables was observed among the accessions (Table 4).

Shoot regeneration was achieved for several accessions on both media with no structures indicative of somatic embryogenesis being detected. Callus growth was not correlated with shoot regeneration. In Protocol 1, shoot regeneration was obtained from 15 accessions, whereas in

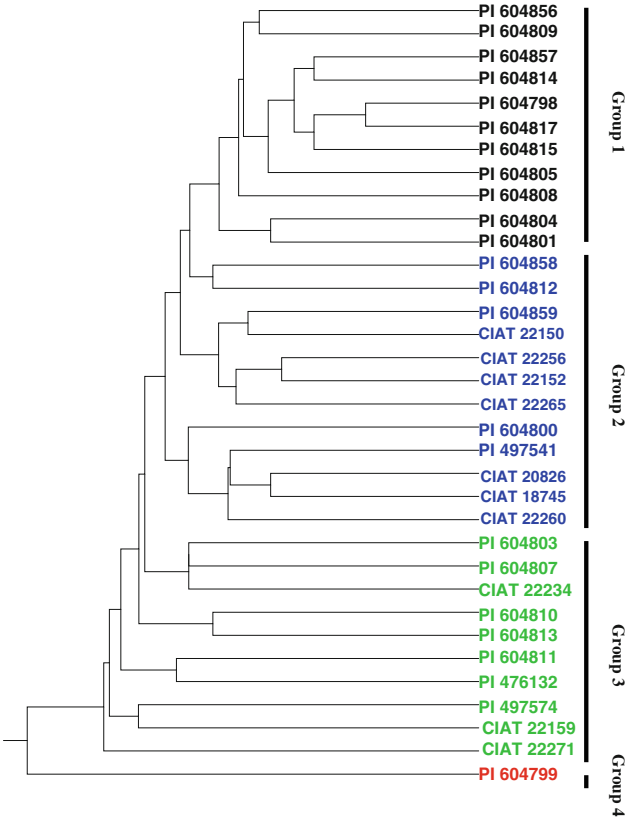

Fig. 1 Dendogram illustrating the genetic relationships among 34 *A. pintoi* accessions based on Nei's genetic distance (Nei 1972) obtained by 100 RAPD markers resolved using eight random primers and generated by the UPGMA method

Protocol 2, shoot regeneration was attained from 18 accessions. Although shoot regeneration was achieved for several accessions as stated before, shoot development and root induction were not achieved in a reliable or repeatable way. Many shoots died during this process. The addition of 1 g l^{-1} of activated charcoal in the root induction medium was tested to evaluate its effect on root formation. However, little or no effect was observed.

Ultimately, 16 regenerated plants were recovered using the two protocols. All plants were phenotypically normal and uniform in appearance, with no somaclonal variation observed visually. Additionally, their RAPD profiles

Table 4 Callus growth rating and weight of *Arachis pintoi* tissue culture incubated in two different protocols

Protocol	Callus growth rating	Callus weight (g)
1	3.10a*	0.333a
2	1.46b	0.078b
Error mean square	0.2868	0.0099

* Means followed by the same letter in the same column were not different by Student-Newman-Keuls's test ($p < 0.05$)

showed no differences when compared to the RAPD profiles of their original parent plants.

Discussion

Genetic analysis

Germplasm characterization is the best way to understand the variability contained in a germplasm collection and to increase use of the germplasm by plant breeders. It is also important in monitoring the genetic stability of the germplasm storage processes. Another positive result of characterization is the detection of duplications, which is a serious problem especially when the size of the collections is large and the resources needed to maintain them are costly. Plucknett et al. (1987) estimated that as little as 35% of the accessions in world collections are actually distinct.

The germplasm of *A. pintoi* contained great variability in gene frequency for each different RAPD locus. According to Nei (1973), when a large number of loci are examined to evaluate the genetic variability of a population, the amount of variation is measured by the proportion of polymorphic loci and average heterozygosity per locus. Also, according to Nei, a locus is called polymorphic when the frequency of the most common allele (x_i) is equal to or less than 0.95 in cases where the sample size is smaller than 50. Using the criterion described above to characterize polymorphic loci, 11 loci were classified as monomorphic. Therefore, we can conclude that 89 of 100 loci (89%) were polymorphic loci.

Although the proportion of polymorphic loci is a good estimation of genetic variability, a more precise and appropriate measure of gene diversity is obtained with gene diversity statistics (Nei 1987). Values for h (0.29 ± 0.16) and H (0.45 ± 0.20) were high, demonstrating that this set of germplasm has a large amount of genetic variability.

Results obtained from this research are equivalent to those reported in the literature for *A. pintoi* with a different set of germplasm accessions and primers. Gimenes et al. (2000) used RAPD markers to evaluate the genetic variation of the *A. pintoi* Brazilian germplasm collection and obtained a total of 104 different bands resolved by ten primers. The average number of bands per primer was 10.4, ranging from 7 to 15. The proportion of polymorphic loci was 90%, and accessions were grouped in three different groups based on their genetic distances.

Bertozo and Valls (1997), also working with a small germplasm collection of *A. pintoi*, reported 220 amplified bands resolved by 22 primers. The average number of bands per primer was 7.5, ranging from 3 to 14 bands. The proportion of polymorphic loci observed was 48.5%. The greatest genetic variability was observed within accessions (0.53), while genetic variability among accessions was reported as 0.39.

RAPDs proved to be a suitable tool to access genetic diversity, relationships among germplasm accessions, and defining the accession identity of the *Arachis pintoi* germplasm. Ten individual accessions were identified by specific RAPD bands. The variables used to assess the genetic diversity of the germplasm indicated that a large amount of genetic diversity exists among the germplasm evaluated in this research. Although a small number of accessions (34) were screened in this research, we can conclude that germplasm collectors have been successful in gathering a group of very genetically distinct individuals.

Tissue culture analysis

Although differences in callus growth and weight among protocols were observed, based on overall shoot development and plant regeneration, both protocols were similar. Several studies have described the developmental problems encountered with organogenic tissue culture protocols of *Leguminosae* species.

Arachis species, among the legumes, have problems with shoot development and root formation. Chengalrayan et al. (1995) obtained on average 33% rooting of shoots in MS hormone-free medium and 52% rooting with NAA in concentrations of 0.01, 0.05, and 0.5 mg l^{-1} added to the medium with *A. hypogaea* cultivar J.L. 24.

Akasaka et al. (2000) were successful in inducing bud formation and shoot development from leaf segments of *A. hypogaea* cv. Chico. The percentage of conversion of buds to shoots was relatively high (34.7%). However, the highest frequency of shoot regeneration was 14.3%, suggesting that some buds failed to grow into normal shoots and plants.

Burtnik and Mroginski (1985), studying methods to regenerate plants from leaf explants of *A. pintoi* cultured on Murashige and Skoog (MS) nutrient medium containing different combinations of NAA (0.1–2 mg l^{-1}) and BA (0.1–3 mg l^{-1}), reported that callus induction was nearly 100%. However, calluses were usually friable and produced no shoots or roots.

Although a small number of plants were regenerated and analyzed, the results indicate that both protocols could be suitable for in vitro plant genetic conservation of *A. pintoi* accessions because no variation in RAPD profiles was observed. Further tests on the regenerated plants must be conducted to assure that no somaclonal variation occurred during the tissue culture process.

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