SCIENTIA
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Análise da capacidade de enraizamento *in vitro* de *Calophyllum brasiliense* utilizando marcadores RAPD

Analysis of *in vitro* rooting capacity of *Calophyllum brasiliense* through RAPD markers

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Resumo

Marcadores RAPD (*Random amplified polymorphic DNA*) são simples, eficientes, baratos e não requerem conhecimento prévio do genoma do organismo estudado, o que é particularmente importante para espécies nativas. O objetivo deste estudo é identificar marcadores moleculares que possam ser usados para caracterizar genótipos responsivos às auxinas usadas para induzir o enraizamento in vitro de explantes de guanandi (*Calophyllum brasiliense*). Amostras de DNA foram extraídas de folhas de plantas mantidas em casa de vegetação e plantas micropropagadas após a fase de enraizamento. Quatro *primers* foram usados e 26 loci encontrados, dois quais 21 são polimórficos (87.5%), com um número de alelos observado de 1.87 e efetivo de 1.39. A diversidade genética de Nei foi 0.24, valor similar ao encontrado em populações naturais. A substituição de nitrogênio líquido por armazenamento a -80°C por meia hora, com a adição de polivinilpirrolidona durante a maceração, foi efetiva na prevenção da degradação das amostras. Os resultados das análises sugerem uma interação entre o genótipo e a resposta à auxina usada durante a cultura *in vitro*, bem como a possibilidade de um grupo de plantas particularmente responsivo. Novas técnicas são necessárias para determinar a presença de um marcador que identifique genótipos mais suscetíveis ao enraizamento *in vitro*.

Palavras-chave: Guanandi, cultura *in vitro*, marcador molecular, polivinilpirrolidona

Abstract

RAPD (Random amplified polymorphic DNA) markers are simple, efficient, inexpensive and do not require prior knowledge of the genome of the organism studied, which is especially important for native species. The objective of this study was to characterize molecular markers that may be used to identify genotypes responsive to the auxin used to induce the *in vitro* rooting of shoots of guanandi (*Calophyllum brasiliense*). DNA samples were extracted from leaves of greenhouse and micropropagated plants after the rooting phase. Four primers were used and 26 loci found, of which 21 were polymorphic (87.5%) with an average number of alleles observed of 1.87 and an effective number of 1.39. Genetic diversity using Nei's measure was 0.24, similar to the values of natural populations. The replacement of liquid nitrogen by storage at -80°C for half an hour, with the addition of polyvinylpyrrolidone during maceration, was effective in preventing the degradation of the samples. The results of the analyses suggest an interaction between the genotype and the response to the auxin used during *in vitro* culture, as well as the possibility of one particular group of responsive arrays. New techniques are needed to determine the presence of a marker that identifies some genotypes more susceptible to *in vitro* rooting.

Keywords: Guanandi, *in vitro* culture, molecular marker, polyvinylpyrrolidone.

INTRODUCTION

Guanandi (*Calophyllum brasiliense*) is an important woody plant belonging to the Clusiaceae family that occurs from Mexico to South America (CARVALHO, 2003; LORENZI, 2008). The species has good quality wood (CARVALHO et al., 2006; SOUZA; LORENZI, 2008) and is indicated for replacement of riparian forest (NERY et al., 2007; COLE et al., 2011). *C. brasiliense* presents several

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medicinal properties (ITO et al., 2002; SOUZA et al., 2009; BRENZAN et al., 2012), including inhibitory action against HIV virus (NOLDIN et al., 2006).The natural reproduction of this species is difficult due to the recalcitrant nature of the seeds (CARVALHO, 2003), low seedling survival (KING, 2003) and poor rooting of cuttings (SILVA, 1998).

A large number of plants can be obtained through *in vitro* culture techniques by using nodal segments excised from greenhouse plants. However, the high genetic variability of mother plants results in great variation of responses when their tissues are cultured *in vitro* and this amenability to *in vitro* culture is considered an inheritable trait (HENRY et al., 1994). *In vitro* responses can therefore be genotype-dependent, as in the case of flax (CHAKRAVARTY; SRIVASTAVA, 1997), cowpea (BRAR et al., 1999), sorghum (ARUSELVI; KRISHNAVENI, 2009) and eggplant (CHAKRAVARTHI et al., 2010). The analysis of the relationship between genotypes and *in vitro* responses is unusual for native forest species, and is primarily restricted to commercial cultivars.

Generally, the genome of South American wild tree species is poorly known. Genetic assays that depend upon this previous genomic information (such as microsatellites or SNPs) are desirable but expensive (when compared to other techniques), time consuming, require sophisticated equipment and well-trained technicians and are sometimes laborious (BARDAKCI, 2001; KUMAR et al., 2009). A prior investigation based on dominant markers is able to recover population level information with less effort and lower costs (KUMAR et al., 2009; LYNCH; MILLIGAN, 1994; SENTHIL KUMAR; GURUSUBRAMANIAN, 2011), as it was the case for *Populus* (RANI et al., 1995), ginger (MOHANTY et al., 2008), cucumber (SMIECH et al., 2008) and apricot (SOLIMAN, 2012). For most of the wild tree species growing in Brazil there is still no possibility of codominant analysis without primer development.

Random Amplified Polymorphic DNA (RAPD) technology (WILLIAMS et al. 1990) is a dominant marker based in decameric oligonucleotides (SENTHIL KUMAR; GURUSUBRAMANIAN, 2011). Due to the small size of the primer, these decamers are able to bind to many genomic loci (KUMAR et al. 2009; SENTHIL KUMAR; GURUSUBRAMANIAN, 2011), allowing polymorphism evaluation. RAPD analysis was once considered not only viable but the most appropriate technique in genotype fingerprinting (WILLIAMS et al. 1990). Nowadays, other several markers are available with better detection power of the genetic variability within populations. As the discriminatory power of the markers is raised, costs and required resources also increase. Nevertheless, suitable statistical treatments are available for dominant markers such as RAPDs (KARP et al., 1997). Thus this marker still holds some strategic advantage for fast population assessments. Some advantages of this technique over other markers involves its operational simplicity, the small amounts of DNA required, the low cost (when compared to other marker technologies) and the fact that no DNA information is required for the design of specific primers (LYNCH; MILLIGAN, 1994; BARDAKCI, 2001; KUMAR et al., 2009; SENTHIL KUMAR; GURUSUBRAMANIAN, 2011).

Dominant markers such as RAPDs, ISSR (Inter-Simple Sequence Repeats) and AFLPs (Amplified Fragment Length Polymorphism) have demonstrated their utility even in population assignment tests (Campbell et al., 2003).

This kind of genetic marker allows preliminary examination of hypotheses related to *in vitro* competency of individuals (HENRY et al., 1994). This approach was previously applied to many species with the same class of genetic dominant marker (RAPD) such as *Populus deltoides* (RANI et al., 1995), *Linum usitatissimum* (CHAKRAVARTY; SRIVASTAVA, 1997), *Vigna unguiculata* (BRAR et al., 1999), *Sorghum bicolor* (ARUSELVI; KRISHNAVENI, 2009), *Solanum melongena* (CHAKRAVARTHI et al., 2010), *Gypsophila paniculata* (BARATAK; EL-SAMMAK, 2011) and *Prunus armeniaca* (SOLIMAN, 2012). In the case of *Calophyllum brasiliense*, RAPD markers were already used to collect basic genetic information on the natural population aiming at the selection of matrices (SCHÜHLI et al., 2013).

As pointed out by Sorin et al. (2006), the failure of cuttings to form adventitious roots occurs routinely and is an obstacle for the clonal propagation and fixation of elite genotypes of woody species. This study aimed therefore to explore this genotyping methodology to search for markers (or a marker set) able to promptly identify auxin responsive specimens. The early recognition of rooting responsive individuals (or genotypes) would prevent enormous laboratory work and greatly improve the efficiency of tissue culture techniques.

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MATERIAL AND METHODS

Samples

Young leaves of 74 3-year-old plants, kept in a greenhouse, and of 120 micropropagated plants were collected and used for DNA analysis. The greenhouse plants also provided nodal segments used as starting material for micropropagation. The leaves of micropropagated plants were collected after the rooting stage, during which they were maintained for 7 days in a culture medium supplemented with indole-butyric acid (IBA) or naphthalene acetic acid (NAA), 10 or 20 µM, and transferred to a growth-regulator-free medium for 60 days. *In vitro* rooted and rootless explants were used for DNA analysis.

Leaf DNA extraction

DNA was extracted from both materials (leaves of greenhouse and micropropagated plants) following the typical CTAB protocol described by Ferreira and Grattapaglia (1998). Some adaptations were made: the original precipitation time at -4°C was increased to 24 h. The micropropagated plants were kept at -80°C (ultrafreezer) for half an hour before maceration instead of liquid nitrogen maceration. To prevent sample oxidation (as pointed out by SAHU et al. 2012) we also included 200 mg of polyvinylpyrrolidone (PVP) during maceration.

For several samples from greenhouse plants, it was not possible to visualize bands on agarose gel, although new extractions were performed. The isolated DNA was a brown or yellowish color. This may indicate a high rate of oxidation of the plant material, as polyphenols and polysaccharides bind firmly to nucleic acids during extraction and interfere with subsequent reactions (PIRTTILÄ et al., 2001). Although the protocol used PVP and βmercaptoethanol, which prevent oxidation of secondary metabolites (PIRTTILÄ et al., 2001), its concentration may have been insufficient to control the oxidation of guanandi leaves.

PVP was effective in controlling oxidation when added during maceration of *in vitro* plants once the presence of bands can be viewed on gel, and white pellets were obtained for all samples. This antioxidant forms hydrogen bonds with polyphenolic compounds, which are subsequently separated by centrifugation (MALIYAKAL, 1992).

During the maceration process, the replacement of liquid nitrogen, a relatively expensive product, by storage of samples in a freezer at -80°C was effective for obtaining high quality DNA. According to Sahu et al. (2012), PVP reduces degradation of DNA.

RAPD analysis

Four primers previously tested within the same species were used to access RAPD profiles. Amplification reactions (PCRs) were performed with the following concentrations for each sample: 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mMdNTPs (each), 0.5 µM primer, 1 unit TaqDNA polymerase, 4 µL of DNA sample (20 ng.μL^{-1}) and ultrapure water to 25 µL (SCHÜHLI et al. 2013).

PCR reactions followed an initial denaturation at 92°C for 4 min, then 40 cycles each consisting of a denaturation at 92°C for 1 min; a 1.5 min annealing at 35°C; and an extension at 72°C for 2 min. A final extension at 72°C for 5 min was applied to achieve full extension of fragments followed by the cooling stage at 4°C (adapted from SCHÜHLI et al., 2013).

Products were stained with bromophenol and ethidium bromide and separated by electrophoresis (1.5% agarose, 80 V, 100 A, 1 to 2 h). A 1 kb ladder was used in gels as reference size marker.

For each sample three independent PCRs and electrophoresis were performed aiming to minimize noise data generated as PCR artifacts. The loci were visually identified and data were coded into a binary matrix (present or absent) containing the 120 individuals from *in vitro* culture (V) and the 74 greenhouse individuals (G). The rooting capability of each sample (for *in vitro* samples) was coded and included in the matrix as a simple binary character.

Tree (dendrogram) computation was processed in PAUP 4b10 (SWOFFORD, 2003) based on Nei Li distances (NEI; LI, 1979). UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) and NJ (Neighbor-Joining), commonly used algorithms for the analyses of natural populations in ecological and taxonomic studies, were used. To estimate genetic similarities among individuals, percentage of polymorphic bands, Nei's gene diversity and Shannon information index Popgene 1:32 were used (YEH et al., 1997).

RESULTS AND DISCUSSION

Four primers resulted in effective amplification, with the number of loci and sequences presented below (Table 1). The data were organized in a binary matrix containing 194 individuals and 26 loci in addition to the characteristic "rooting" in the case of *in vitro* plants. We found 21 (87.5%) polymorphic loci with an average number of observed alleles per loci of 1.87 and effective alleles of 1.39. Nei's genetic diversity index was 0.24, a value similar to that found in the natural environment (SCHÜHLI et al., 2013) and Shanon information index was 0.56. The OPE 20 primer amplified few loci (3), while the OPC 9 resulted in 10 (Table 1).

Table 1. Primers used for polymerase chain reaction (PCR) of leaves of *Calophyllum brasiliense*, according to the nucleotide sequences and the number of loci.
Tabela 1. Primers utilizados na reação em cadeia de polimerase (PCR) de folhas de *Calophyllum brasiliense*, de acordo com as sequências de nucleotídeos e número de loci.

Primer	Sequence (5'-3')	Loci number
OPA 01	CACGCCCTTC	7
OPC 10	TGTCTGGGTG	9
OPE 20	AACGGTGACC	3
OPJ 19	GGACACCACT	7
Total	-	26

UPGMA algorithm

With the UPGMA algorithm, samples of rooted plants 69V, 93V and 102V were grouped together with 8V and 24V (Figure 1, red branches). During the rooting stage of micropropagation, the 8V and 24V plants were cultured in a medium containing 10 µM indolebutyric acid (IBA), while samples 69V, 93V and 102V were exposed to 10 µM 1-naphthaleneacetic acid (NAA). Only plants exposed to NAA rooted at *in vitro* stages.

This result may suggest genotypic differences related to the explants' responses during the rooting stage in the presence of an auxin. As pointed out by Sorin et al. (2006), adventitious rooting is known to be a genetic trait. Similarly, specific cultivars of flax had better rates of shoot bud initiation during organogenesis, showing a genotype-related response (CHAKRAVARTY; SRIVASTAVA, 1997). According to Gahan and George (2008), the plant genotype has a marked effect on the behavior of plant tissues, so it is possible to obtain plants with improved performance in all aspects of culture. In the case of eggplant seeds, for example, seven genotypes tested showed variation in their *in vitro* responses, with increased productivity of some specific genotypes (CHAKRAVARTHI et al. 2010). This relationship between genotypes and *in vitro* responsiveness is well documented for several commercial cultivars such as maize, barley and rice (as reviewed by XOCONOSTLE-CÁZARES, 2011). For guanandi, however, the absence of genotypes with a sequenced genome limits the identification of lineages potentially valuable for micropropagation.

These results also suggest that the rooting capacity can be the result of an interaction between the genotype and the culture conditions, since unrooted individuals (8V and 24 V) were exposed to a different auxin (IBA) during the rooting phase. According to Chakravarthi et al. (2010), several intrinsic and extrinsic factors influence tissue culture responses, especially the nature and age of the explant, genotype, type and concentration of growth regulators. Different varieties and plant genotypes may differ in their requirements for growth regulators, particularly auxin, in the culture medium (GEORGE, 2008). The genetic basis of responsiveness to *in vitro* culture relies on the genes involved in plant hormone metabolism (HENRY et al., 1994). This indicates that the performance of plant material during *in vitro* culture is related to its ability to respond to hormonal stimuli received at each stage of *in vitro* culture.

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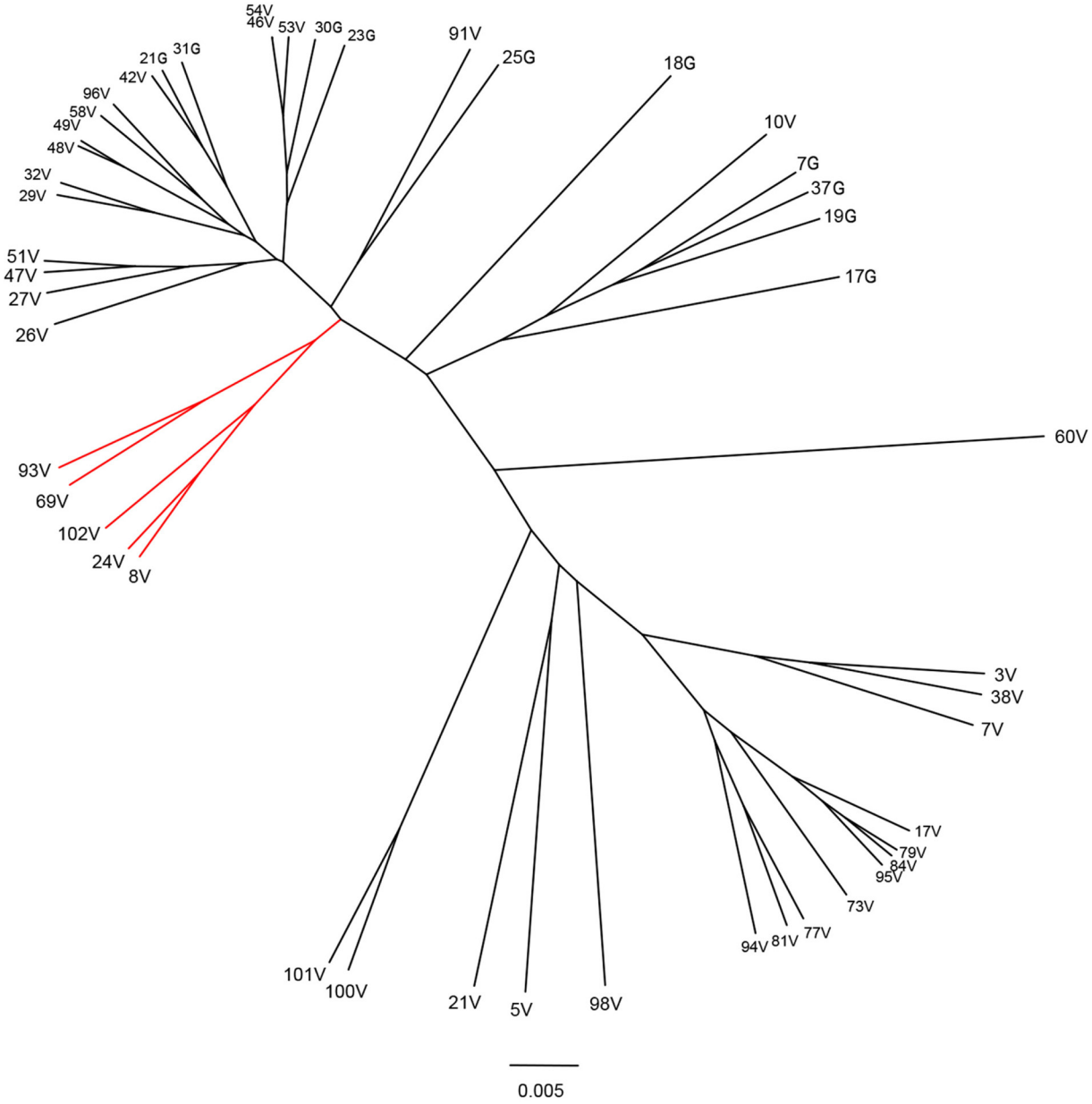

Figure 1. Unrooted dendrogram representing genetic relationships, using distance analysis (UPGMA). Branch length represents the genetic distance (NEI; LI, 1979) among individuals. V – *in vitro* plant; G – greenhouse plant.
Figura 1. Dendrograma não enraizado representando relações genéticas, utilizando análise de distância (UPGMA). O comprimento dos ramos representa a distância genética (NEI; LI, 1979) entre os indivíduos. V - planta cultivada *in vitro*; G - planta de casa de vegetação.

NJ algorithm

In the same way as the UPGMA analysis, the neighbor-joining algorithm grouped together the rooted plants 69V and 93V, which again suggests a relationship between these two individuals (Figure 2, branches in red). The more closely related group contains the samples 18G, 21G and 42V, the first two being from greenhouse matrices. The 42V plant was also rooted *in vitro*. The proximity with this greenhouse plant may indicate a particularly responsive genotype for *in vitro* rooting. The presence of a genetic marker could be suggested, or a pattern of bands that can be used as an indicator for *in vitro* rooting response or greater sensitivity to the auxin added to the culture medium. This approach has been proposed to identify RFLP markers linked to tissue culture response of potato (COLEMAN et al., 1990). A SCAR (sequence-characterized amplified region) marker has already proved to be related to high rooting ability for larch cuttings (LI et al. 2008). Likewise, various RAPD and SSR (simple sequence repeats) markers were associated with rooting traits (such as length, secondary roots and dry weight) for coffee (ACHAR et al. 2015).

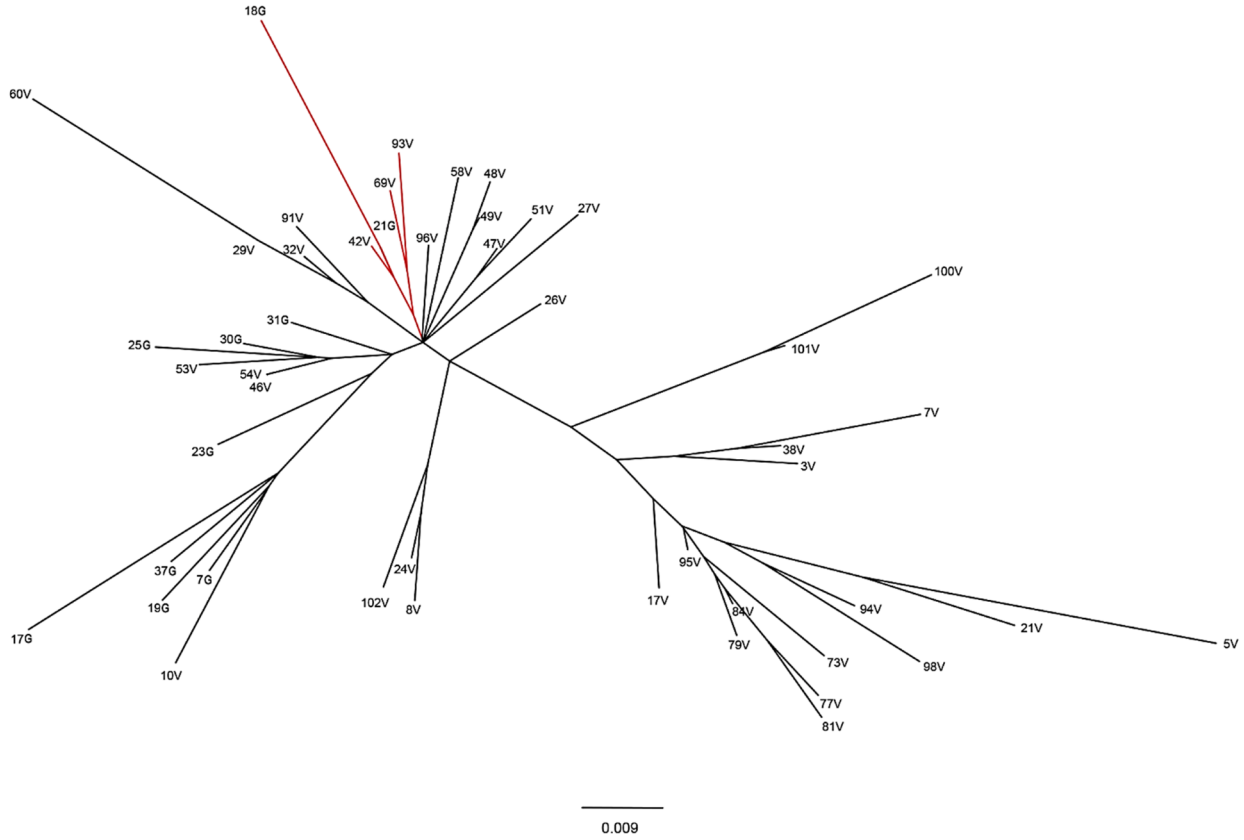

Figure 2. Unrooted dendrogram representing genetic relationships, using Neighbor-Joining analysis. Branch length represents the genetic distance (NEI; LI, 1979) among individuals. V – *in vitro* plants; G – greenhouse plants.
Figura 2. Dendrograma não enraizado representando as relações genéticas, usando análise de Agrupamento de Vizinhos. O comprimento dos ramos representa a distância genética (NEI; LI, 1979) entre indivíduos. V - planta cultivada *in vitro*; G - planta de casa de vegetação.

The grouped samples (18G, 21 G, 42V, 69V and 93 V) have nearly 55% similarity among the observed bands, suggesting the need for further analysis with new individuals to determine whether such a marker exists. If this marker is found, band isolation on agarose gel, purification of DNA sample, cloning by PCR, and finally its sequencing can be performed in order to obtain a specific primer to determine the predisposition to responsiveness during micropropagation. As pointed out by Gahan and George (2008), once a selection method is established, it is possible to improve the results obtained from *in vitro* cultures by conventional plant breeding techniques.

CONCLUSIONS

It is possible to characterize the guanandi germplasm of *in vitro* and greenhouse plants using RAPD markers. The refinement of purification techniques is important for obtaining high quality DNA for molecular genotyping analysis and processing. The oxidation and subsequent DNA degradation can be minimized by the addition of PVP during the maceration of the leaves.

Some results suggest that the RAPD technique is able to retrieve information on the characteristics of the material during *in vitro* culture, as well as specific genotypes of matrices that have greater responsiveness and are preferable for vegetative propagation. If validated, these indicative loci can be used as markers of superior individuals having the necessary characteristics for *in vitro* rooting. In the future, more specific primers may be included and more specific analysis conducted in order to refine the results obtained in this study.

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