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Clonal Fidelity and Genetic Diversity of Micropropagated *Hancornia speciosa* Gomes (Apocynaceae) as Evaluated by Molecular Markers

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Abstract: The plant tissue culture procedures for asexual multiplication of *Hancornia speciosa* represent an important process facilitating the preservation of selected genotypes of this threatened species and large-scale production of plantlets for population reinforcement or other utilization. However, there is no research regarding the somaclonal variation of this threatened species at molecular level. This study aimed to evaluate the role of the multiplication process in the genetic stability of *H. speciosa* plantlets and calluses using molecular markers. The tissue culture techniques for *H. speciosa* micropropagation used in this study did not influence the occurrence of somaclonal variation, which can be related to the genotypes of the donor plants and to the low concentrations of growth regulators used in tissue culture procedures adopted. The results observed here provide a reliable methodology for further studies involving micropropagation techniques with the goal of preserving selected or endangered genotypes of *H. speciosa* without genetic losses and producing seedlings of this species on a large scale. In addition, it was possible to verify that the donor plants used in this study showed a relatively low genetic variability.

Keywords: genetic stability; ISSR; micropropagation; organogenesis; SSR

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1. Introduction

Hancornia speciosa Gomes (Mangaba) is a fruit tree belonging to the Apocynaceae family, widely distributed in savannas and open vegetation from the northeast towards central–west Brazil, Paraguay, Bolivia, and Peru [1,2]. It is an intermediate-sized tree with an average height ranging from 4 to 7 m and can reach up to 15 m [3]. The species is described as hermaphroditic and self-incompatible, requiring different genotypes for cross-pollination, which leads to a high genetic variability [3–5]. The edible fruit are considered flavored and aromatic and can be consumed in natura or used as raw material for candy, ice cream, and juice production, playing an important role in local markets [3,6,7]. Moreover, it produces abundant latex that has been used to treat diabetes [8] and for rubber production [9,10]. Despite its economic potential, *H. speciosa* fruits are mostly collected from nature (extractivism) since there are few orchards, which are impaired by the presence of recalcitrant seeds with a high loss of viability when stored [11,12]. The extractive harvesting of fruits (and seeds); recalcitrant seeds, which make it difficult to propagate the tree [13]; and fragmentation of habitats due to increased environmental degradation and anthropogenic action [14–16] are resulting in a decrease in natural occurrence areas of *H. speciosa* and contributing to the loss of genetic variability [13,15,17,18]. Thus, conservation strategies should be developed for this species [6,19].

Plant tissue culture has been widely used for asexual multiplication, leading to the conservation of selected or threatened genotypes to produce seedlings on a large scale [12,20,21]. Among many techniques, micropropagation is noteworthy since it can be

used commercially, allowing a plant with desirable characteristics to be clonally multiplied in a short time with a reduced space and in adequate phytosanitary conditions [22–24].

Nevertheless, tissue culture systems that involve the acquisition of competence for totipotency and extensive cell division have greater tendency to genome and epigenome instabilities, which has been termed as somaclonal variation (SV) [25,26]. SV is a major concern in all in vitro vegetative plant propagation systems because it leads to the loss of genetic fidelity [27]. The causes of SV are not well understood and have not been fully elucidated [28]. Some factors that determine the frequency of SV include the in vitro propagation method, the type and concentration of growth regulators applied, and the number and length of subcultures [26,28,29].

Although SV can be used as a source of variability by plant breeders with relative success, the loss of clonal fidelity has hampered commercial micropropagation. SV is detrimental for commercial purposes when the main objective is strictly clonal propagation of elite plant material to ensure the maintenance of selected traits [26,30,31]. Hence, tools for identifying SV should be used to ensure the genetic fidelity of micropropagated plantlets. Several approaches have been widely used to detect the occurrence of SV, such as chromosomal analysis, flow cytometry, and DNA fingerprinting. Among these, the molecular markers (e.g., simple sequence repeats—SSR—and inter-simple sequence repeats—ISSR) are independent of environmental influences, showing reliability and repeatability of the data and a high degree of polymorphism [32–36].

SSR markers, also known as microsatellites, have gained great importance in the evaluation of genetic stability due to their desirable characteristics such as high reproducibility, co-dominance, a high level of polymorphism that can be detected, and their technical facilities [37]. By using polymerase chain reaction (PCR) with specific primers for the regions of the DNA that flank the microsatellites, it is possible to amplify these regions. Therefore, to perform work involving the use of SSR markers, the development of specific primers for the species under study is necessary [37].

On the other hand, ISSR markers are DNA fragments amplified by PCR using unique primers designed from microsatellite sequences amplified by PCR. As there is no need for prior knowledge of DNA sequence data to design the primer used, ISSR markers can be used as a viable alternative for plant species that have not yet developed specific primers in addition to having the advantage of generation of large numbers of informational bands per reaction [15,18,37].

Despite efforts to develop a reliable protocol for in vitro establishment and shoot multiplication for *H. speciosa* [12,20,38–40], there is no research regarding the SV at the molecular level focused on this species. Accessing the genetic stability of plantlets recovered by tissue culture techniques at early stage through molecular markers is recommended since morphological variations in plants raised through tissue culture can be detected usually at later stages [41]. Therefore, this study aimed to evaluate the effect of subculturing on SV rate in *H. speciosa* plantlets and calluses using ISSR and SSR molecular markers. In addition, a genetic diversity analysis of the donor *H. speciosa* plants was carried out.

2. Materials and Methods

2.1. Plant Material

The plant material was obtained from ripe fruits (light yellow color with reddish streaks and tender texture) of *H. speciosa*, which was sampled in forest fragments considering a distance of 30 to 50 m between sampled trees in October of 2016 at the three different sites: (1) Site 1: Gameleira farm, located in Montes Claros de Goiás–GO (19°53′ S and 44°25′ W); (2) Site 2: Jatobá farm, in Caçu (18°781′ S and 52°58,307′ W); and (3) Site 3: Água Amarela farm, in Ouroana (18°11′ S and 50°34′ W), Brazil.

2.2. In Vitro Establishment and Propagation

The fruit pulp removal was performed manually in an aluminum mesh sieve (number 30). The seeds were manually washed under running water to remove the tegument and

submersed for 10 min in a container with running water with three drops of Tween (80%). Then, they were immersed in a bowl with 70% (*v*/*v*) alcohol for 1 min and in a solution of sodium hypochlorite (20%) for 20 min, followed by a triple wash with autoclaved distilled water in a laminar flow, as described by [38]. For in vitro establishment, the seeds were germinated in glass tubes (25 × 150 mm) containing 20 mL on half-strength Murashige and Skoog salts ($\frac{1}{2}$ MS [42]) supplemented with vitamins [43], 30 g L^{-1} sucrose (Sigma®) (St. Louis, MO, USA) and 3.5 g L^{-1} agar (Sigma®) (St. Louis, MO, USA). The pH of media was adjusted to 5.7 ± 0.03 prior to autoclaving. Culture tubes were maintained under a photoperiod of 16 h at 25 ± 3 °C, a relative humidity of 45%–46%, and an active photosynthetic radiation of 45–55 mol m^{-2} s^{-1} [38]. Once the in vitro establishment was completed (about 60 days), these plants were used as explants donors for further procedures (Figure 1).

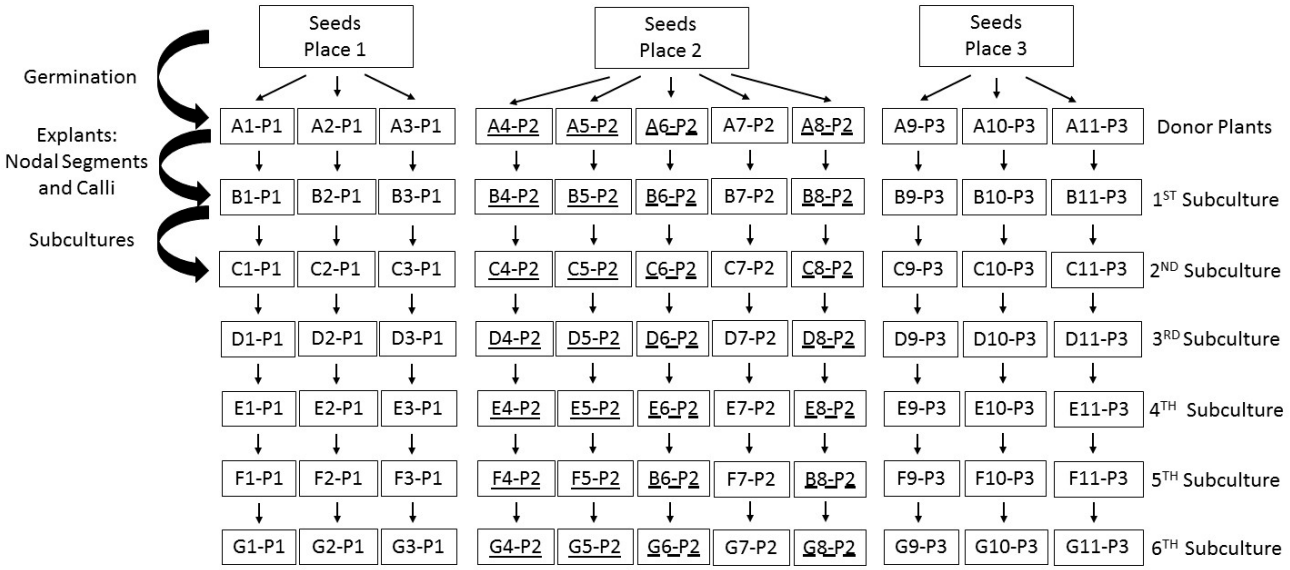

Figure 1. Schematic illustration of the plant individuals analyzed in this study. The first generation of plants (Group A) was obtained from in vitro germination of seeds of donor plants from Montes Claros, GO (Site 1); Caçu, GO (Site 2); and Ouroana, GO (Site 3). These plants provided donate nodal segments explants and calluses (Group B, donor plants). The first, second, third, fourth, fifth, and sixth subcultures (Groups C–G) were produced from explants donated by plants of group B. Plants used only as callus donor explants are shown underlined, and donors used as nodal segment explants are shown with a dash line. Plants with no underlining were used as donors for explants for both experiments (from [35] with adaptation).

Nodal segments from in vitro germinated seeds were used as explants in subsequent subcultures to evaluate the effect of this procedure on SV rate. Each nodal segment explant was 2 cm in length and had two lateral buds. These explants were transferred to glass flasks (70 × 100 mm) with a plastic cap containing 40 mL of the described MS $\frac{1}{2}$ culture medium. The flasks containing four nodal segments remained for 30 days under the same growth conditions described above, allowing shoot multiplication. Subcultures were performed every 30 days, totaling six subcultures at the end.

As an alternative pathway to obtaining explants for micropropagation approaches (e.g., indirect embryogenesis or organogenesis), a procedure for obtaining calluses was tested. Leaves from in-vitro-germinated plantlets were removed, shared in two parts, and cultivated in glass flasks (12 × 50 mm) containing callus induction medium, which consisted of the MS $\frac{1}{2}$ culture medium supplemented with vitamins [43], 30 g L^{-1} sucrose (Sigma®) (St. Louis, MO, USA), 3.5 g L-1 agar (Sigma®) (St. Louis, MO, USA), 6-benzylaminopurine (BAP, 1 mg L^{-1}) (Sigma®) (St. Louis, MO, USA), and naphthalene acetic acid (NAA, 2.5 mg L^{-1}) (Sigma®) (St. Louis, MO, USA). After the formation of calluses (60 days), they were replaced

to a fresh media every 30 days, performing six subcultures. The plant tissue culture assays were conducted at Laboratory of Plant Tissue Culture at IF Goiano–Campus Rio Verde, GO and followed the *H. speciosa* tissue culture protocols established by Cabral et al. [32].

2.3. DNA Extraction and Molecular Markers' (SSR and ISSR) Amplifications

The plant material recovered from tissue culture was divided into groups for extraction of genomic DNA, following the methodology adopted by [35]. In this study, Group A corresponds to eleven in-vitro-established plants used as explant donors (from Sites 1, 2 and 3) for in vitro propagation. Group B was composed of eleven plantlets from nodal explants of Group A. Groups C, D, E, F, and G consisted of another eleven plantlets from nodal explants of Groups B, C, D, E, and F, respectively, following the same procedure for all six subcultures (Figure 1). For the identification of calluses after each subculture, the same procedure was adopted.

Total genomic DNA was extracted from leaves of donor plants and their respective shoots (derived from nodal segments) or calluses (derived from leaves of donor plants) collected at each performed subculture, following standard procedures [38,44]. The concentration and quality of the extracted DNA was assessed via determination of A260/A280 absorbance ratio using a spectrophotometer (NanoDrop™, Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). DNA purity were estimated via 0.8% agarose gel electrophoresis (Loccus Biotecnologia, Brazil) stained with GelRed™ (Biotium, Hayward, CA, USA).

For SSR amplifications, 16 microsatellite loci developed by [45] were selected for the assessment of clonal fidelity between donor plant and in vitro regenerated shoots or calluses. For the ISSR approaches, we selected 15 primers that produced reproducible alleles (Table 1; commercialized by University of British Columbia, Vancouver, Canada) after the preliminary screening of 20 ISSR primers [7,46,47].

Table 1. General characteristics of ISSR and SSR primers selected for evaluation of somaclonal variation and genetic diversity in *Hancornia speciosa* plants.

Primers	Sequences (5′–3′)	AT [1]	NB [2]	NPB [3]	P (%) [4]	PIC [5]
UBC 1	ACACACACACACACACT	50.0	5	3	60.0	0.281
UBC 2	GAGAGAGAGAGAGAGAT	50.0	4	1	25.0	0.124
UBC 808	AGAGAGAGAGAGAGAGC	52.4	4	1	25.0	0.117
UBC 809	AGA GAG AGA GAG AGA GG	52.4	5	2	40.0	0.100
UBC 810	GAGAGAGAGAGAGAGAT	50.0	3	1	33.3	0.060
UBC 812	GAG AGA GAG AGA GAG AA	50.0	7	1	14.3	0.063
UBC 815	CTC TTC TCT CTC TCT CTG	53.9	5	0	0.0	0.000
UBC 818	CAC ACA CAC ACA CAC AG	52.4	3	1	33.3	0.165
UBC 825	ACA CAC ACA CAC ACA CT	50.0	5	2	40.0	0.158
UBC 826	ACACACACACACACACC	52.4	3	1	33.3	0.060
UBC 834	AGA GAG AGA GAG AGA GYT	51.6	5	1	20.0	0.069
UBC 848	CAC ACA CAC ACA CAC ARG	53.9	8	6	75.0	0.169
UBC 851	GTG TGT GTG TGT GTG TYG	53.9	5	0	0.0	0.000
UBC 855	ACA CAC ACA CAC ACA CYT	51.6	4	1	25.0	0.105
UBC 866	CTC CTCCTCCTCCTCCTC	60.7	4	0	0.0	0.000
	Total	-	70	21	-	-
	Average	-	4.66	1.4	28.2	0.098

[1] Annealing temperature; [2] number of bands; [3] number of polymorphic bands; [4] percentage of polymorphism; [5] polymorphic informational content. Y = (C, T).

DNA amplification was performed in a total volume of 25 µL, with 120 ng of template DNA, 1× PCR buffer (5 mM de MgCl2, 50 mM de Tris/HCl (pH 9.0), 50 mM de NaCl), 0.2 mM dNTP mix (Sigma®) (St. Louis, MO, USA), and 1.25 U Taq DNA polymerase (GoTaq®, Promega, Madison, WI, USA). Both forward and reverse microsatellite primers (Table 2) were added to a final concentration of 1 µM. The samples were amplified in a

thermal cycler (BioRad T100 TM) using following the program: initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50–61 °C (according to each primer, Table 2) for 1 min and extension at 72 °C for 1 min with a final extension at 72 °C for 7 min.

Table 2. General characteristics of SSR primers selected for evaluation of somaclonal variation and genetic diversity in *Hancornia speciosa* plants.

Primers	Sequences (5′–3′)	AT [1]	NA [2]	PIC [3]
HS 01	F: GTGTCTTCCATCCGAGCTTAAC R: TTTCCCAGAAAGGAGAGGTACA	50	3	0.593
HS 11	F: GTGATATTTCGTGCTCTCCAAG R: CTCTGCCACTGTGCAACC	50	2	0.180
HS 18	F: ATTCATGCTCCACTGGCTTC R: GACCACAGCTAGTGACGTGTTC	50	2	0.500
HS 17	F: ACTCGAGCAGAAGAAGCAAATC R: ACACACCCTCATCAGCCC	54	2	0.500
HS 27	F: TATAGTGGTCCTGCACCCTTGT R: TTTTCCCTTGTGCTTCGC	54	2	0.278
HS 08	F: AATGTAGAGGTGAACGAGTGGG R: TACACCCTGCTCATCGTTTATG	48	1	0.000
HS 16	F: CGTTGGTAGCGGCTGTATTAAG R: CCCCTCCTGCCACTCTCT	48	2	0.500
HS 10	F: ACAAATCAATGAGGAGGTGCTT R: TAACTATGTGCAACCGCAAGAC	52	3	0.583
HS 13	F: CTGGGGTACTTCAGCAAATCAC R: CATCAAAGACCGTTGTCTCCTT	56	1	0.000
HS 14	F: GAGCAGGAGTCAGGAAAATCAC R: ACAGTGAAGGGGCAATGAAG	56	3	0.525
HS 22	F: GGACGAAACGAAATGGAGAGTA R: AGTAAAGACACGTCATCCCCAC	56	1	0.000
HS 23	F: TGCAAACCCTCATTTCTTTTCTTC R: GGAGCAAATCGGGAAGCC	56	2	0.500
HS 30	F: GAGGAATCTCAGCCAAGTCCTA R: CCCAGCCTCTACAAACTCTCTG	56	2	0.500
HS 33	F: CGTTGGTAGCGGCTGTATTAAG R: CACTCTCTTTTCCCGATTTTCC	56	3	0.665
HS 24	F: GCTAAATCAAGCAAACCTCGAC R: AAAGCAGTCCATGATCCATTTC	58	2	0.463
HS 26	F: CAAACAAGCTTTATGTGGGTCA R: AGCTCAAGGAAGTGGGATCTAA	58	1	0.000
	Total		36	
	Average		2	0.362

[1] Annealing temperature; [2] number of alleles; [3] polymorphic informational content.

PCR products were visualized on 1.5% (*w*/*v*) agarose (Sigma®) (St. Louis, MO, USA) gel using 1× TBE buffer and stained with GelRedTM (Biotium, Fremont, CA, USA), then visualized and photographed using the gel documentation system L.PIX fitted with an 8 b CCD camera and UV light (Loccus Biotecnologia, São Paulo, SP, Brazil). The allele sizes were calculated by comparing with 100 bp DNA ladder (Promega, Madison, WI, USA). At least two independent PCR amplifications were performed for each primer. The

molecular markers approaches were carried out at the Laboratory of Biotechnology, at IF Goiano–Campus Rio Verde, GO.

2.4. Data Analysis

The subcultured nodal segments and calluses and their respective donor plant were compared to detect to identify possible somaclonal variants. For this purpose, only consistently reproducible and well-resolved bands were manually scored. For genetic diversity analysis, the ISSR band profiles were processed as dominant markers and each *locus* was considered as a bi-allelic locus with one amplifiable and one null allele. Data were scored as 1 for the presence and 0 for the absence of a DNA band for each locus. For SSR analysis, a matrix containing 1, 2, and 3 was generated according to the number of alleles for each locus, where coding 11, 22, and 33 refers to homozygotes and 12, 13, and 23 to heterozygotes. Genetic dissimilarity was estimated based on the complement of Jaccard and weighted indexes using the GENES software [48]. The generated dissimilarity matrix was used to calculate the frequency of dissimilarity, and Unweighted Paired Group Method using Arithmetic Averages (UPGMA) was used to obtain the dendrogram. The cophenetic correlation coefficient (CCC) between the matrix of genetic dissimilarity and the matrix of cophenetic values was estimated using the RStudio 3.5.1 (R Development Core Team version 2020, Vienna, Austria) to verify the clustering consistency. The polymorphic information content (PIC) was calculated for each primer, in order to estimate the efficiency of the primers in the indication of polymorphism between two individuals. For the binary data, the PIC was calculated using the following equation:

$$\text{PICi} = \sum 2\text{fi}\ (1 - \text{fi}), \quad (1)$$

in which fi is the frequency of the amplified allele (band presence). For codominant data (SSR), the following equation was used:

$$\text{PIC} = 1 - \sum P_{ij}^2 \quad (2)$$

in which P_i is the frequency of the *i*-th allele of the *j*-th loci. Principal Coordinates Analysis (PCoA) was carried out using the RStudio 3.5.1 (R Development Core Team version 2020, Vienna, Austria) Vegan package.

3. Results

3.1. In Vitro Propagation

High seed germination percentage was observed without fungal or bacterial contamination, generating well-formed and vigorous seedlings, exhibiting stem and leaves with a dark-green color and well-developed adventitious roots after 60 days (Figure 2A). These materials were used as explant sources for the subsequent procedures involving the direct growth of nodal segments and the calluses' induction.

In vitro development of the nodal segments to plantlets occurred about 15 days of culture on half-strength basal MS medium (Figure 2B). The length of the inoculated shoots was nearly 2 cm and after the subculture, each shoot formed 4–10 dark-green leaves. The formation of axillary shoots was found occasionally. In the early subcultures, a well-developed adventitious root system was observed. However, as the subcultures were performed, the nodal segments did not develop further roots.

Conversely, the first callogenic response signals were observed on the margins of the leaf explants of *H. speciosa* after 30 days in medium (Figure 2C). Subsequently, the formation of calluses expanded from the margin towards the central region of the explants, and after another 30 days, the calluses were completely formed (Figure 2D). Thus, on the 60th day after cultivation in the medium containing BAP and NAA, the leaf explants were responsive to callus induction treatment.

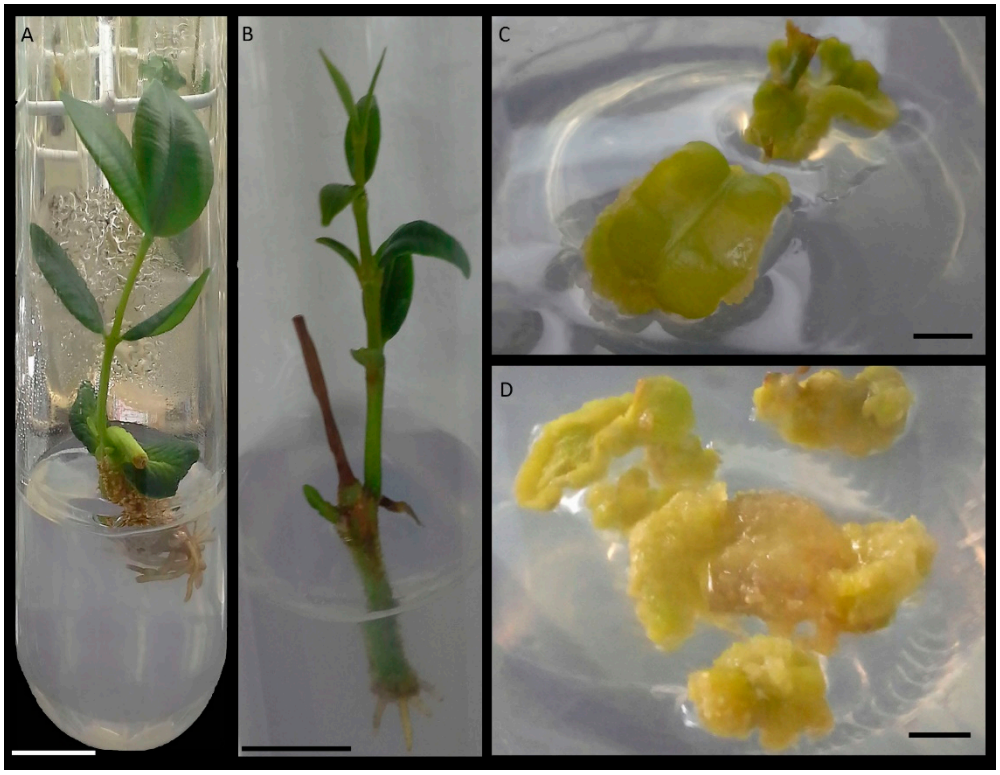

Figure 2. Representative illustration of *Hancornia speciosa* in vitro establishment and multiplication by nodal segments and callus induction. (**A**) In-vitro-established seedlings of *H. speciosa*. (**B**) Shoot formation from nodal segments. (**C**) *H. speciosa* starting callus formation. (**D**) *H. speciosa* callus after subcultures. Bar = 1 cm.

The results obtained in the present study indicate that the protocol used for in vitro propagation of *H. speciosa* nodal segments was adequate for regeneration of plantlets. Additionally, the culture media tested for callus induction was adequate and can be used in future indirect organogenesis approaches.

3.2. Analysis of Somaclonal Variation Using Molecular Markers

The use of molecular markers allowed a rapid evaluation of the occurrence of alterations at DNA level along of six subcultures of nodal segments and calluses. Band pattern analysis generated by SSR and ISSR markers did not reveal the presence of SV during the six subcultures performed when compared to donor plants (Figure 3).

Analysis of the produced bands pattern revealed polymorphisms between the donor plants for both primers. From 15 selected ISSR primers, 12 were polymorphic (80%) and 3 were monomorphic (20%), obtaining a total of 70 amplified bands with 21 being polymorphic (Table 1). UBC 848 primer provided the highest total number of bands and the largest number of polymorphic bands (eight and six, respectively). The polymorphism percentage (P%) ranged from 0 (UBC 851, UBC 815, and UBC 866), up to 75.0% (UBC 848), with a general primer average of 28.2%. The polymorphism information content (PIC) ranged from 0 (UBC 851, UBC 815, and UBC 866) up to 0.281 (UBC 1), with an average of 0.098.

Regarding the SSR marker analysis, 16 primers were selected, resulting in a total of 32 alleles amplified, with an average of two alleles per primer (Table 2). HS 01, HS 10, HS 14, and HS 33 primers presented the highest number of alleles (three alleles each). The polymorphic information content (PIC) ranged from 0 (HS 08, HS 13, HS 22, and HS 26) to 0.665 for (HS 33), with an average of 0.361.

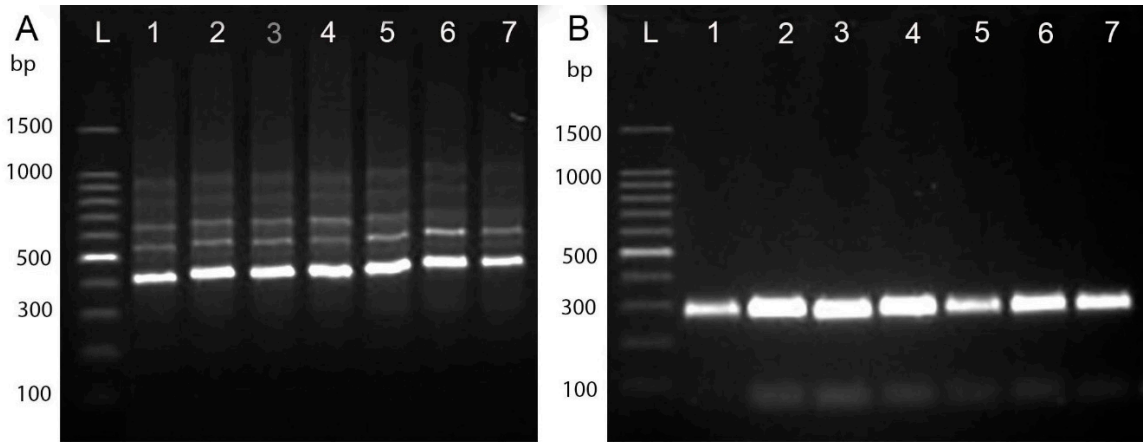

Figure 3. Examples of DNA profiles amplified from micropropagated *Hancornia speciosa* plantlets by using ISSR primer UBC 851 (**A**) and SSR primer HS 08 (**B**). L: 100 bp DNA ladder from Invitrogen. 1: ISSR (**A**) and SSR (**B**) profiles for the donor plant A5, from Site 2 (Caçu). Lanes 2 to 7 represent tissue culture recovered from plants during six subcultures.

3.3. Genetic Diversity Analysis

As no SV was observed in the subcultured plantlets when compared to donor plants, dissimilarity analysis was performed only on the donor plants of the threesites.

For ISSR primers, the Jaccard Index complement was used to obtain the matrix of dissimilarity between the donor plants from Montes Claros, GO (Site 1); Caçu, GO (Site 2); and Ouroana, GO (Site 3). It was observed that the minimum value of dissimilarity was found between A1-P1 and A3-P1 (0.016), and the maximum value of dissimilarity (0.174) was between A10-P3 and A8-P2 (Table 3). The lowest mean of individual dissimilarity was obtained by A1-P1 and the highest by A10-P3—with 0.069 and 0.105, respectively—and the average among all donor plant matrices was 0.089.

The dissimilarity matrix among the individuals (donor plants), using the data of the SSR primers, was obtained by the complement of the weighted index. The lowest dissimilarities observed were between A4-P2 and A5-P2, A10-P3, and A11-P3, with 0.000, and the greatest dissimilarities occurred between A8-P2 and A10-P3, A8-P2, and A11-P3, with 0.344 (Table 3). The lowest mean individual dissimilarity was obtained by A7-P2 and the highest by A3-P1, with 0.127 and 0.215, respectively, and the average among all donor plants was 0.174.

Pearson's correlation between the dissimilarity matrices of ISSR and SSR was positive, of median magnitude (0.510), and significant at 1% probability according to the *t* test, which shows agreement between the two matrices.

Genetic dissimilarity values obtained by the different molecular markers showed a low dissimilarity value, indicating that the donor plants were genetically close.

According to the results of UPGMA cluster analysis from the ISSR markers, a dendrogram was constructed. A2-P1 and A9-P3 were removed from this analysis due to the amount of data lost. From the cut-off point, the donor plants were discemed into two clusters: Group I was formed by plants from Site 2 (Caçu), and Group II was formed by plants from Site 1 (Montes Claros) and Site 3 (Ouroana) (Figure 4A). For SSR markers data, UPGMA clustering distributed the donor plants matrices into three groups from the cut-off point: Group I was formed by plants from Site 2 (Caçu), Group II was formed by plants collected in Site 1 (Montes Claros), and Group III was formed by Site 3 plants (Ouroana) (Figure 4B). Thus, the use of molecular markers ISSR and SSR allowed us to cluster the donors from the same sites, demonstrating the efficiency of these markers in quantifying the genetic variability of the *H. speciosa* genotypes at the molecular level.

Table 3. Genetic dissimilarity values between pairs of individuals from the different *Hancornia speciosa* donor plants using the complement of Jaccard and weighted indexes for ISSR (above the diagonal) and SSR markers (under the diagonal), respectively.

	A1-P1	**A3-P1**	**A6-P2**	**A7-P2**	**A8-P2**	**A4-P2**	**A5-P2**	**A10-P3**	**A11-P3**	
A1-P1	0	0.02	0.10	0.13	0.13	0.10	0.07	0.07	0.03	
A3-P1	0.14	0	0.09	0.13	0.15	0.12	0.12	0.09	0.05	
A6-P2	0.19	0.28	0	0.08	0.09	0.12	0.11	0.15	0.10	
A7-P2	0.14	0.23	0.05	0	0.05	0.08	0.09	0.16	0.15	
A8-P2	0.17	0.25	0.17	0.13	0	0.06	0.08	0.17	0.16	
A4-P2	0.17	0.20	0.13	0.08	0.20	0	0.03	0.15	0.13	
A5-P2	0.17	0.20	0.13	0.08	0.20	0.00	0	0.12	0.11	
A10-P3	0.22	0.31	0.27	0.22	0.34	0.25	0.25	0	0.05	
A11-P3	0.22	0.31	0.27	0.22	0.34	0.25	0.25	0.00	0	
ISSR A [1]	0.07	0.08	0.09	0.09	0.10	0.09	0.08	0.11	0.09	0.09
SSR A [2]	0.16	0.22	0.16	0.13	0.20	0.14	0.14	0.21	0.21	0.17

[1] Average ISSR markers; [2] average SSR markers.

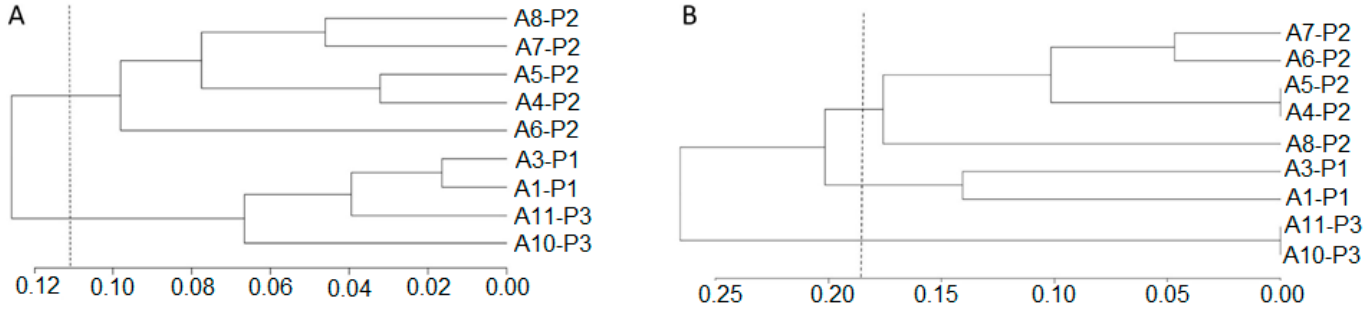

Figure 4. Genetic similarity dendrogram obtained by the UPGMA cluster analysis from ISSR data (Jaccard Index, (**A**)) and SSR data (weighted index, (**B**)) band patterns of different *Hancornia speciosa* donor plants collected in Montes Claros (A1-P1 and A3-P1), Caçu (A6-P2, A7-P2, A8-P2, A4-P2, and A5-P2), and Ouroana (A10-P3 and A11-P3).

The cophenetic correlation coefficient (CCC), which is a measure of agreement between the original dissimilarity values and those represented by the dendrogram (the higher the CCC the lower the grouping distortion) was calculated. The obtained CCC was 0.844 and 0.899 for ISSR and SSR, respectively, representing a high fit between the co-phenic matrix and the dissimilarity matrix and demonstrating high cluster reliability.

PCoA analysis was performed to evaluate the distribution of the nine selected *H. speciosa* genotypes in the ordination space, with the x-axis representing 75.68% and 73.61% of total genetic variation for the dissimilarity matrices obtained with ISSR and SSR data, respectively (Figure 5).

According to the PCoA using ISSR markers, two clusters were discemed: (i) GI1 containing the genotypes A1-P1, A3-P1, A11-P3, and A10-P3 and (ii) GI2, formed by A6-P2, A8-P2 and A7-P2, A5-P2, and A4-P2 (Figure 5A). Using SSR data, three clusters were formed: (i) GS1, which contains A7-P2, A5-P2, A4-P2, and A6-P2 genotypes; (ii) GS2, with A1-P1, A8-P2, and A3-P1; and finally, GS3, consisting of A10-P3 and A11-P3.

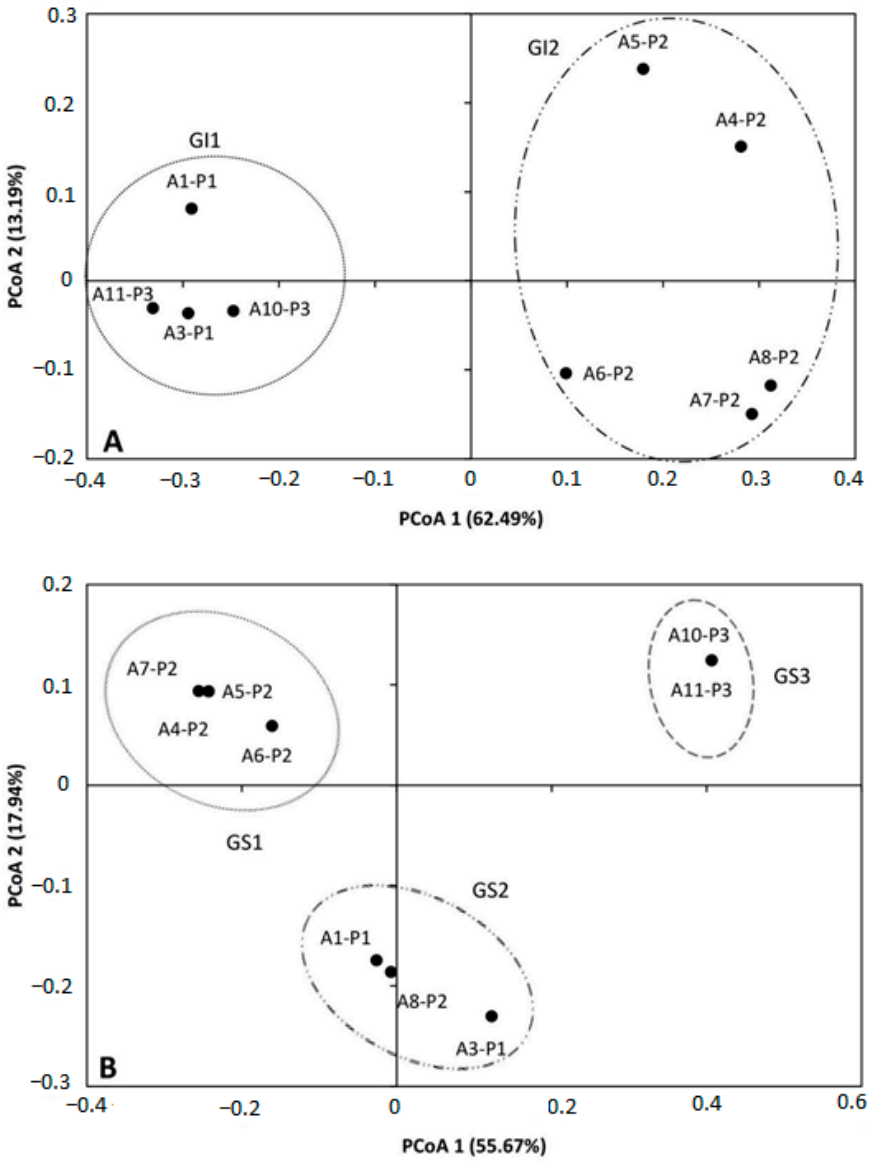

Figure 5. Principal Coordinates Analysis (PCoA) obtained by the complement of Jaccard Index for ISSR data (**A**) and by the complement of weighted index for SSR data (**B**) for *Hancornia speciosa* genotypes collected in Montes Claros (A1-P1 and A3-P1), Caçu (A6-P2, A7-P2, A8-P2, A4-P2 and A5-P2), and Ouroana (A10-P3 and A11-P3). The dotted lines highlight the clusters formed by GI1 e GI2 using ISSR data (**A**) and GS1, GS2, and GS3 for SSR data (**B**).

4. Discussion

4.1. Analysis of Somaclonal Variation Using Molecular Markers

Although the SV causes have not been completely elucidated, the conditions that trigger this phenomenon during micropropagation are related to stress factors such as wounding, exposure to sterilizing agents, use of undifferentiated cells, concentrations of sugar, light conditions, and hyperhydricity (vitrification) [49]. The type of explant used for in vitro cultivation [50], and in vitro environment conditions have been described as determinant conditions for SV occurrence [25]. In vitro environment is frequently understood as the type and concentration of plant growth regulators (PGR) [25,51], the growth medium conditions (e.g., solid, semi-solid or liquid/immersion system) [52], as well as consecutive subcultures [51]. Moreover, the regenerative pathway (direct versus indirect, organogenesis, or embryogenesis) has been related with the induction of SV [53].

Whereas there is no study of SV at the molecular level in *H. speciosa*, we conducted this study to access the genetic stability of *H. speciosa* plantlets submitted to different pathways (direct organogenesis from nodal segments and calluses), during six subcultures. Our results showed that no SV was observed in the in vitro cultivated nodal segments or calluses when compared to donor plants, indicating the genetic stability along the six subcultures. The absence of SV is desirable, since the loss of clonal fidelity has been a problem for commercial micropropagation of elite plant material to ensure the maintenance of selected traits [26,30,31]. Thus, the methodologies employed here were suitable to micropropagation of *H. speciosa* plants, giving rise to plants identical with the donor genotypes.

In the present study, we observed genetic stability with nodal segments or calluses explants. According to Kamenická and Rypák [54], plantlets recovered by the organogenetic pathway show higher genetic stability when compared with those obtained after calluses induction. Furthermore, regeneration of plants through unorganized callus formation [55] is frequently related to the occurrence of SV, as the indirect pathway requires callus formation and the plant cells are directed to dedifferentiation pathways that can be a source of mutations. As revised by Bairu et al. [28], a disorganized growth phase in plant tissue culture is one factor that may cause SV, as in vitro conditions can be stressful to plant cells and often trigger genetic variations. However, it is also possible to find SV in axillary buds [56] since the stabilizing influence of the meristem is usually lost when plants are submitted to in vitro conditions [28,57].

One possible explanation for the absence of SV observed is that we used axillary buds and young leaves (obtained from in vitro germinated seeds) as explants during the subcultures and for callus induction. These tissues were not highly differentiated and were probably less prone to SV [58,59]. Another condition that may have contributed to the absence of SV in the regenerated *H. speciosa* in vitro recovered plants in this study was the low concentrations of PGR employed. Excessive PGR concentration in culture media can be a cause of SV in micropropagated plants [25,28]. Relatively high concentrations of N6-benzyladenine (BA) have been related with SV in banana [60], and SV was shown in strawberry [61], soybean [62], and cotton [63] subjected to 2,4-D treatments. Moreover, there are some reports showing contradictory results, leading to the role of PGR as a cause of SV being uncovered.

Regarding the frequency of subcultures as a cause of SV, an increase inthe number and duration of subcultures would be expected to increase the rate of SV [28]. In face of this, the absence of SV found herein could relate with the relatively few numbers of subcultures performed in this study. However, other studies [33] report SV after the fourth callus subculture of *Tetrastigma hemsleyanum* using enzyme activity analysis and molecular markers (ISSR and SRAP). This indicates that in the above-mentioned case, only four subcultures can be performed to obtain plants identical to the donor plant. The opposite trend (no SV) is reported in another study with sugarcane after 15 subcultures [64]. Therefore, a possible explanation for the absence of SV verified in the present study may be the genetic constitution of the individuals since each genotype responds differently to the variation that can be caused by the stress during the in vitro cultivation with more stable genotypes than others due to these differences in genetic composition [28,57].

The use of ISSR and SSR markers allowed the evaluation of the genetic fidelity, stability, and true-to-type of *H. speciosa* calluses and nodal explants along six subcultures. Various DNA-based molecular markers have been proposed to assess clonal fidelity in micropropagated plants, leading to the inference of SV. Moreover, different authors have shown the reliability of molecular markers to assess SV in different plants and types of explants, suggesting that the use of different molecular markers allow to access several loci, which increases the probability of SV identification [65–67] without environmental influence [68]. While ISSR and SSR markers have been used for monitoring clonal fidelity in a large number of plant species, there is no study of SV at molecular level in *H. speciosa*. Studies carried out with micropropagated sugarcane, such as [69], found no SV analyzing nine cultivars through the use of RAPD markers. The same is true of [62], which used ISSR

markers to analyze two cultivars during 15 subcultures. These results are in agreement with the results obtained in the present study, in which no SV was identified in the analyzed loci. Other studies using ISSR markers have revealed clonal fidelity of in-vitro-micropropagated *Gerbera jamesonii* Bolus ex Hook.f. plants (all of them derived of three explants), reporting only one clone with SV and all the others identical to the donor plants [70]. The authors reported only one clone exhibiting SV, and all the others were identical to the donor plants. Likewise, 91% clonal fidelity has been reported in 20 *Platanus acerifolia* plants which were subcultured every two months for eight years [71]. More recently, 98% genetic similarity has been found using RAPD markers among 14 *Aloe vera* plantlets in in vitro conditions subjected to six months of subcultures [72]. According to Martinez [73], somaclonal variation is influenced by the reproductive biology of the species, the number of individuals taken as tissue source, and the tissue culture protocol, while its detection and measurement depends upon the molecular marker system selected, which can also vary in the intensity of genome sampling.

4.2. Genetic Diversity Analysis

According to Martínez [73], the knowledge of preexistent genetic variability is necessary to be able to estimate genetic variations caused by in vitro tissue culture techniques, since different reproductive conditions lead to variable genetic diversity. Moreover, Żabicki et al. [74] recommended to perform a genetic variation assessment before reintroduction of in vitro raised plants for conservational purposes. Since *H. speciosa* shows a cross pollination and self-incompatible reproductive biology, we should expect a high genetic divergency among individuals. However, our analysis using ISSR and SSR markers revealed a low polymorphism between individuals, similar results in *H. speciosa* with SRR were observed by Bogea et al. [75] and Soares et al. [76] and with ISSR by Costa et al. [46], Luz et al. [18] and Santos et al. [77]. These results can be explained by the fact that samples are taken from forest fragments close to farms (great anthropic activity), which can lead to a small number of specimens of the species in these places and to the successive crossing between related plants, leading to a decrease in genetic diversity within the region's population and can cause depression through inbreeding. According to Darrault and Schlindwein [78], *H. speciosa* are pollinated by different species of bees, moths, and butterflies; thus, the insecticides used to control pests in agricultural crops can kill these pollinators, decreasing the pollination of populations close to farms.

PIC values range from 0 for monomorphic profiles to 1 for highly polymorphic profiles [79]. According to this classification, a PIC value below 0.25 indicates low polymorphism, a value between 0.25 and 0.5 indicates average polymorphism and a value above 0.5 indicates a highly polymorphic locus. In this study, all ISSR locus amplified can be considered low and average polymorphic, hence, low informative (Table 1). For SSR amplified loci, the polymorphism information was considered low informative for markers HS 11, HS 08, HS 13, HS 22, and HS 26; average for markers HS 18, HS 17, HS 27, HS 16, HS 23, HS 30, and HS 24; and finally, highly informative for markers HS 01, HS 10, HS 14, and HS 33 (Table 2).

Evaluating herein the genetic diversity of 15 individuals from a natural *H. speciosa* population (Rio Grande do Norte, Brazil) using ISSR markers, a total of 63 loci were amplified; only 30 (47.62%) were polymorphic, and PIC values ranged from 0.26 to 0.44, with an average value of 0.37, indicating a low genetic diversity within the population [46]. On the other hand, Jimenez et al. [7] analyzed 38 *H. speciosa* individuals belonging to natural populations in Pernambuco, Brazil and generated 93 ISSR loci, 10 (10.8%) monomorphic and 83 (89.255%) polymorphic, demonstrating high genetic variability in that population. More recently, Silva et al. [80] evaluated the genetic diversity of 35 individuals from three localities of "Restinga" vegetation (Ceará and Pernambuco), using nine ISSR primers and obtained 61 completely polymorphic amplicons (100%), with a higher percentage of polymorphic loci for the three locations (85.25%, 77.05%, and 59.02%), and PIC average values ranging from 0.43 to 0.78.

Nunes et al. [15], studying the genetic diversity of a natural population of fourteen trees of *H. speciosa* in the coastal region of the state of Sergipe, observed 81 polymorphic loci obtained from 17 ISSR primers that were amplified with 100% polymorphism—the average number of fragments per primer was 7.88—and concluded that this population has high genetic diversity. On the other hand, [18] evaluated the genetic diversity of *H. speciosa* populations from the Embrapa Meio-Norte Germplasm Bank using ISSR indicators. A total of 29 accessions from Brazil were characterized and the presence of low or medium genetic diversity were identified.

CCC values above 0.8 are an indication of good representability between distances [81]. The obtained CCCs were 0.844 and 0.899 for ISSR and SSR, respectively, representing a close fit between the cophenic matrix and the dissimilarity matrix, demonstrating high cluster reliability.

Comparing the clusters obtained by PCoA analysis (Figure 5A,B), we observed that clusters are different according to the molecular marker data used. Conversely, when the cluster formed by the PCoAs (ISSR and SSR, Figure 5A,B, respectively) are compared with those showed in the dendrograms (ISSR and SSR, Figure 4A,B, respectively) we found very similar distribution of the genotypes within the clusters formed, demonstrating that the difference in the formed groups is due to the nature of the types of markers used.

Evaluating the genetic structure and inter- and intra-populational genetic diversity of natural populations of *H. speciosa* using molecular markers ISSR by means of PCoA, we observed that the first two main coordinates explained 67.77% of the total observed variation [47]. Also evaluating the diversity of *H. speciosa* populations with the aid of molecular markers ISSR, reported that the first two main coordinates explained 72.00% of the variation [82]. These results are inferior to those found in this work, demonstrating a greater precision in the PCoA cluster found in the present study.

Thus, low dissimilarity was observed among used donor plants, especially for those collected at the same sites, since they obtained the lowest values of dissimilarity and were grouped in the same group in the dendrogram. Therefore, the donor plants used in this study are genetically similar—that is, they have low genetic variability between them.

5. Conclusions

The tissue culture techniques for *H. speciosa* micropropagation used in this study did not influence the occurrence of SV. This observation can probably be related to the genotype and to the low concentrations of growth regulators used in the adopted tissue culture procedures. The lack of SV shows that the micropropagation of *H. speciosa* plants in the conditions described can be successfully carried out, maintaining genetic fidelity with the donor plant in the progeny up to the sixth subculture. Also, this study confirms the utility of ISSR and SSR markers for monitoring SV in early stages. As there is no previous report of SV monitoring of in-vitro-propagated *H. speciosa* along subcultures, the results observed provide a reliable methodology for further studies involving micropropagation techniques with the goal of preserving selected or threatened genotypes and producing seedlings of this species on a large scale. In addition, it was possible to verify that donor plants used in this study showed relatively low genetic variability. Thus, in order to use micropropagation techniques to preserve the species, it is suggested that individuals with higher genetic variability be selected.

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Data Availability Statement: Original data presented in the study are included in the main text, and further inquiries can be directed to the corresponding author.

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