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Genome Status of *Lippia alba* Polyploid Complex Long-term *in vitro* Cultivated

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Authors' contributions

This work was carried out in collaboration among all authors. Author SAJ raised the in vitro plants for the experiments. Authors SAJ, JMLL, CZ and EMM conceived, designed and performed the experiments. Author LFV contributed to the design and interpretation of the research and to the writing of the paper. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

This study is the first report of a genetic stability analysis of a polyploid complex maintained *in vitro* for a long-time. Twenty-two accessions of *Lippia alba*, a medicinal species of economic importance, had been maintained under *in vitro* culture conditions for 7 years through sprouting of axillary buds. Four clones of each accession were analyzed, being three plants from *in vitro* bank and one cultivated in the field. We investigated the genetic stability of diploid, aneuploid, triploid, tetraploid, and hexaploid accessions. The investigation was carried out using flow cytometry, inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers. No significant variation in nuclear DNA content was observed between the *in vitro* conserved plants and their respective field plant. Out of 23 ISSR primers screened, 8 primers were found to produce clear reproducible bands resulting in a total of 5456 bands. 86.36% of the analyzed plantlets (19 accessions) showed at least one polymorphic band. The polymorphic rate ranged from 1.61 to 33.87%. The SSR markers were

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used to confirm the absence or low occurrence of variation in accessions that showed no polymorphism or polymorphism for only one ISSR primer. The genetic instability detected in our study at the molecular level may be attributed to the natural instability of *L. alba* genome combined with the long-time *in vitro* maintenance.

Keywords: Flow cytometry; in vitro culture; somaclonal variation; ISSR; SSR; verbenaceae.

1. INTRODUCTION

The *in vitro* technique is a powerful tool to maintain plant genetic resources. It has been widely used for a long time as an efficient alternative to preserve native and cultivated plant germplasm [1,2] and also to quickly increase the number of plants of commercial interest [3]. In addition, the possibility to modulate the production of chemical components constitutes an interesting aspect to explore *in vitro* cultivation [4,5,6]. One of the crucial aspects of *in vitro* culture is the maintenance of plant genome integrity. During development, the explants might be exposed to particular conditions provoking the destabilization of the genetic and epigenetic program of plant tissues leading to chromosomal and DNA variations, also known as somaclonal variation [7]. The *in vitro* differentiation and the frequency of eventual genetic variation can be affected by different factors such as media composition, culture conditions, pattern of regeneration, plant stress, species genetic background, and number of subcultures [8,9,10].

Although, *in vitro* culture has been successfully used for a long-time as an alternative to keep the germplasm safe from field issues in different species [11,12], no reports about the genetic stability of a polyploid series after long-term *in vitro* maintenance has been published so far. Thus, the influence of genome size after long-term *in vitro* propagation is underexplored.

Flow cytometry and molecular markers have been widely used to analyze the genetic stability of regenerated plantlets [8,13,14]. Among the most used markers, ISSR and SSR have been largely used due to its simplicity, speed, and cost-effectiveness being highly discriminative and reliable [15,16,17,18]. These markers have been very useful to investigate the genetic stability of several micropropagated species such as *Gossypium hirsutum* [15], *Platanus acerifolia* [19], *Alhagi maurorum* [20], *Gerbera jamesonii* [16], *Pisum sativum* [21], *Zea mays* [17], *Bacopa monnieri* [14], and *Achras sapota* [22].

Lippia alba (Mill.) N.E.Brown (Verbenaceae) is an aromatic herb native to South and Central America [23]. In Brazil, the species is widely found throughout the country and is popularly known as 'erva-cidreira' [24,25,26]. Due to the medicinal properties, the species has been used for a long time in folk medicine as a sedative, tranquilizer, analgesic, anxiolytic, antispasmodic, expectorant and to treat digestive, respiratory, and cardiovascular problems [23,26]. *L. alba* is considered as one of the mostly used plant in Brazilian folk medicine being one of the 66 regulated species with medicinal purposes [26].

This species possess an extraordinary chemical [23], morphological [27], and genomic variation [28,29]. Due to the high variability in the chemical composition of the essential oil of *L. alba*, the species has been extensively studied regarding the essential oil production, chemical composition, biological activities, etc. [25,30,31, 32,33,34]. Few studies have been done about biological aspects of the species [28,35,36,37, 38]. Five chromosome numbers were reported [28] raising the importance to investigate the genetic variation of the species using different approaches. Curiously, *L. alba* polyploid complex showed an interesting association between chemical profile and ploidal level, opening new possibilities to explore and modulate the production of essential oil. Linalool, an important component to the perfume industry, is efficiently produced by triploids while diploids and tetraploids are citral producers [37].

In spite of the importance of the species, few studies have investigated aspects of the *in vitro* cultivation of *L. alba*, focusing mainly in the plant regeneration response either from the apical/axillary buds [24,39,40,41,42,43] or leaf-derived calli [44]. To date, no study on *L. alba in vitro* cultivation has assessed the genetic fidelity of long-term micropropagated plants. Here, we investigate the genetic stability of diploid, aneuploid, triploid, tetraploid, and hexaploid accessions of *L. alba* cultivated *in vitro* for 7 years. The investigation was carried out using flow cytometry technique and molecular markers (ISSR and SSR). Our results provide the

first documentation of the genetic profile of the species under long-term *in vitro* maintenance conditions.

2. MATERIALS AND METHODS

2.1 Plant Material

Twenty-two accessions of *L. alba* with different ploidal levels (diploid, triploid, tetraploid, hexaploid, and aneuploid) were collected in different Brazilian states (EMS1). The accessions were established *in vitro* from axillary meristems in 2008. Each accession was also propagated in the field at Plant Experimental Station, Universidade Federal de Juiz de Fora (UFJF), Minas Gerais State, Brazil, and *in vitro* in the Laboratory of Genetics and Biotechnology, at the Institute of Biological Science, Universidade Federal de Juiz de Fora, MG, Brazil. *in vitro* plantlets were maintained in test tubes containing 15 mL of MS-based medium devoid of growth regulators and subcultivated on a bimonthly. Three replicates of each accession were kept on this medium for 7 years at 25 ± 2ºC under a light regime of 16/8 h (light/dark) cycle of 35 µmol m^{-2} s^{-1} illumination provided by cool, white fluorescent tubes. The accessions were grouped according to the ploidal levels (see Fig. 1 for details). Voucher specimens were deposited at Herbarium Leopoldo Krieger (CESJ-UFJF), Minas Gerais, Brazil.

2.2 Genetic Stability

For cytometric and molecular analysis, young and fresh leaves for each accession of *L. alba* were sampled.

2.2.1 Flow cytometry analysis

To estimate the DNA content of *L. alba* accessions cultivated in field and *in vitro*, approximately 30 mg of leaf were macerated with the same mass of the internal reference standard *Pisum sativum* L. cv. 'Ctirad' (2C = 9.09 pg). The nuclei extraction was performed by chopping the leaves in a Petri dish containing 1 mL of cold LB01 buffer to release the nuclei into suspension [45]. The homogenate was filtered and the nuclei subsequently stained with 25 µL of propidium iodide (10 mg/L), with 2.5 µL of RNAse (20 mg/L) added to each sample. At least 10000 nuclei were analyzed per sample in a FACSCanto II (Becton Dickinson) flow cytometer. Each output flow cytometric histogram obtained from FACS Diva 6.1.3 software was analyzed using Flowing 2.5.1 software (available at http://www.flowingsoftware.com/). The nuclear DNA amount (pg) of each sample was estimated by the relative fluorescence intensity of the sample and the internal reference standard (*Pisum sativum* 9.09 pg) according to Doležel, et al. [46]. Each sample was estimated three times.

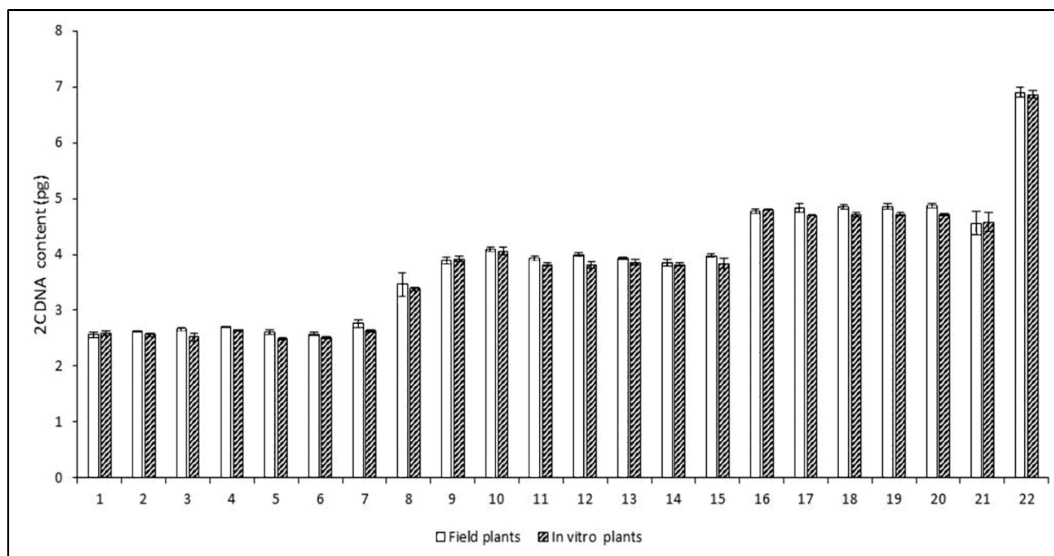

Fig. 1. Nuclear DNA content of field plants and seven years *in vitro* maintained accessions of *L. alba*. Accessions 1-7: diploid (2*n* = 30), 8: aneuploid (2*n* = 38), 9-15: triploid (2*n* = 45), 16-21: tetraploid (2*n* = 60), 22: hexaploid (2*n* = 90)

The effect of *in vitro* culture time on the DNA content was evaluated individually for each accession comparing the DNA amount of the *in vitro* plantlets with the same genotype cultivated in the field. The DNA content of field plants was pairwise compared with the values obtained for *in vitro* plantlets. Differences in genome size were assessed by the chi-square test at $p < 0.05$.

2.2.2 Isolation of genomic DNA

Total DNA was extracted from *in vitro* and field samples using the cetyltrimethylammonium bromide (CTAB) method as described by Doyle and Doyle [47] with minor modifications. Four samples of each accession were analyzed, three from plants cultivated *in vitro* for seven years and one from a field plant. In total, 88 samples were analyzed. DNA quality and yield was checked by 1% agarose gel electrophoresis and Nanodrop^{TM} 2000 (Thermo Scientific) spectrophotometer.

2.2.3 ISSR analysis

Twenty three ISSR primers were initially screened and the eight most polymorphic primers were selected. Each PCR reaction was performed in a total volume of 25 µL reaction containing 0.5 µM primer, 0.15 mM dNTPs, 1 unit of Taq DNA polymerase, 10 mM Tris-HCl (pH 8,0), 2 mM MgCl2 and 30 ng of DNA. DNA amplifications were performed in an Eppendorf MasterCycle thermocycler, with the following conditions: initial denaturation at 94ºC for 4 min, followed by 45 cycles of 60s at 94ºC, 45s at annealing temperature, 2 min extension step at 72ºC and a final extension at 72ºC for 7 min. The annealing temperature was adjusted according to each primer. DNA amplified products were loaded on a 2% agarose gel and submitted to electrophoresis using 1×TBE buffer (Tris-Borate-EDTA buffer) stained with 0.1 µL/mL of SYBR® Safe DNA gel stain and photodocumented with UV transilluminator UVP GelDoc-It Imaging-Vision Works LS system. The molecular weight of fragments was estimated using a molecular marker of 100 bp.

2.2.4 SSR analysis

SSR analysis was done to support some particular results of ISSR pattern obtained for five accessions. ISSR results that showed genetic variation for less than two primers were assessed by microsatellite markers. SSR primers specifically developed for *L. alba* were used (Lopes, et al. in prep.). After a preliminary screening of 16 primers, the five most informative primers were selected. The PCR reactions were carried out in a volume of 10 µL 1X GoTaq® buffer, 0.013 mM forward primer, 0.5 mM reverse primer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 unit of Taq DNA polymerase and 30 ng of genomic DNA. DNA amplification was conducted using the following conditions: initial denaturation at 94ºC for 3 min, followed by 35 cycles at 94ºC for 45s, annealing for 45s, extension at 72ºC for 45s and final 20 min extension at 72ºC. DNA amplification products were separated by electrophoresis in 8% polyacrylamide gels using vertical electrophoresis chambers with Tris-Borate-EDTA (TBE) electrode buffer. The amplification products were visually analyzed and the molecular weight of the fragments was estimated using a molecular marker of 50 bp.

2.2.5 Data analysis

The amplification reaction for all primers was repeated twice to confirm the reproducibility of the results. To quantify the genetic polymorphism, the PCR amplification patterns were recorded as a binary matrix, in which the presence or absence of fragments with similar size (for ISSR and SSR markers) were scored as 1 or 0, respectively. Only consistent, reproducible and well resolved bands, ranging from 100 to 1500 bp in size (for ISSR and SSR markers), were included in the analysis. The variation was expressed, considering new or absent bands, comparing each *in vitro* sample with the respectively field plant.

3. RESULTS

3.1 Vegetative Growth Performance

L. alba accessions were successfully *in vitro* propagated in a growth regulator free medium using nodal segments. The axillary dormant buds sprouted within 5–10 days after inoculation. The rooting process occurred simultaneously with sprouting after 7–15 days. After 2 months, each *in vitro* propagated explant of all accessions produced at least eight nodes per plantlet. Insignificant differences among the accessions were observed regarding *in vitro* response. In general, the senescence process started after 2 months of maintenance.

3.2 Genome Size

The simultaneous analysis of *L. alba* nuclear suspensions and the internal reference standard showed a typical two-peak profile (G0/G1, G2/M)

with low CV (below 5%). The absence of additional peaks demonstrates the ploidy stability of the accessions cultivated *in vitro* for a long-time. In the analysis of the DNA content, no significant difference (*P < 0.05*) between *in vitro* conserved plants and respective field plant was observed (Fig. 1).

3.3 Molecular Analysis

Eight ISSR primers were selected after a preliminary screening of 23 ISSR primers. All ISSR markers yielded 62 scorable and reproducible bands with an average of 7.75 bands per ISSR primer. Each primer produced a unique set of amplification products ranging in size from 200 to 1600 bp. The number of bands for each primer varied from 4 (UBC-820) to 11 (UBC-809; Table 1). 5456 bands (number of samples analyzed times the number of scorable bands considering all 8 primers) were generated by ISSR markers (Table 1). Of these bands, 145 were polymorphic across all 88 plants tested.

Polymorphism between *in vitro* and *ex vitro* plants was noted when the fragment was present/absent in the field plant, but absent/present in the *in vitro* clones (see Fig. 2 for an example). The number of polymorphic bands was variable. Some regenerated plants accumulated a large number of polymorphic bands, whereas few plants had no polymorphic band (accessions 16, 17, and 21). At least one polymorphic band was present in 86.36% of the analyzed plantlets (19 accessions) and the number of polymorphic bands among *in vitro* and field plants ranged from zero to 21 depending on the accession. The polymorphic rate ranged from zero to 33.87% (Table 2). Accessions 18 and 22 showed genetic variation for only one primer, with one and two polymorphic fragments, respectively. Only one accession revealed polymorphism for all primers, also showing the highest polymorphic rate.

Curiously, the number of polymorphic bands decreased as the ploidal level increased. Diploids showed 69 polymorphic bands, whereas triploids and tetraploids exhibited 49 and 20 polymorphic bands, respectively.

The accessions that showed no polymorphism (16, 17, and 21) or polymorphism for only one ISSR primer (18 and 22) were also analyzed using microsatellite markers to confirm the absence or low occurrence of variation. Out of 16 SSR markers specific for *L. alba*, five were selected due to the most informative profile. The amplification of these five SSR markers produced 32 reproducible fragments. Microsatellite fragments ranged from 100 to 400 bp in size. The number of bands varied from 2 (Primers SSR-294 and 473) to 12 (Primer SSR-229), and the average number of bands per primer was 6.4. The 640 bands (number of samples analyzed times the number of scorable bands considering all five primers) generated by the SSR markers were monomorphic (data not shown) reinforcing the results obtained by the ISSR markers.

Table 1. Primer and amplified fragments information generated by ISSR and SSR markers of long term *in vitro* cultured (7 years) and field cultivated plants of *Lippia alba*

Primer	Total bands	Range of bands	Size range (bp)
ISSR			
UBC-809	11	3-11	300-1000
UBC-835	10	4-8	250-1300
UBC-854	5	1-4	300-1400
UBC-818	7	3-7	400-1600
UBC-820	4	2-4	600-1200
UBC-830	8	3-7	300-1500
UBC-848	9	2-8	200-1000
UBC-850	8	3-6	300-1000
SSR			
77	7	3-5	140-250
294	2	1-2	160-190
229	12	7-9	140-250
440	9	4-9	150-400
473	2	1-2	100-120

Table 2. Polymorphism of ISSR data comparing long term *in vitro* cultivated plantlets (7 years) with the control plants (field cultivated) of 22 accessions of *Lippia alba*

Accessions[a]	Primer								Number of primers detecting polymorphisms	Total polymorphic bands	Polymorphic rate (%)
	Number of polymorphic/total bands										
	UBC-830	UBC-848	UBC-850	UBC-820	UBC-818	UBC-854	UBC-835	UBC-809			
1	1/8	3/9	0/8	0/4	0/7	1/5	3/10	0/11	4	8	12.90
2	2/8	1/9	3/8	1/4	1/7	1/5	3/10	2/11	8	14	22.58
3	0/8	1/9	0/8	0/4	0/7	1/5	0/10	0/11	2	2	3.23
4	2/8	3/9	3/8	0/4	2/7	1/5	3/10	5/11	7	19	30.65
5	0/8	1/9	0/8	0/4	1/7	1/5	0/10	0/11	3	3	4.84
6	1/8	2/9	1/8	1/4	0/7	1/5	2/10	1/11	7	9	14.52
7	2/8	2/9	2/8	0/4	0/7	1/5	6/10	1/11	6	14	22.58
8	1/8	2/9	0/8	0/4	0/7	2/5	0/10	0/11	3	5	8.06
9	0/8	1/9	1/8	0/4	0/7	2/5	0/10	0/11	3	4	6.45
10	3/8	1/9	0/8	0/4	0/7	0/5	0/10	0/11	2	4	6.45
11	0/8	1/9	0/8	0/4	0/7	1/5	0/10	0/11	2	2	3.23
12	1/8	0/9	0/8	0/4	0/7	2/5	0/10	0/11	2	3	4.84
13	2/8	3/9	0/8	0/4	0/7	2/5	0/10	0/11	3	7	11.29
14	1/8	4/9	0/8	0/4	0/7	3/5	0/10	0/11	3	8	12.90
15	3/8	5/9	3/8	0/4	0/7	3/5	2/10	5/11	6	21	33.87
16	0/8	0/9	0/8	0/4	0/7	0/5	0/10	0/11	0	0	0.00
17	0/8	0/9	0/8	0/4	0/7	0/5	0/10	0/11	0	0	0.00
18	0/8	0/9	0/8	0/4	1/7	0/5	0/10	0/11	1	1	1.61
19	2/8	0/9	2/8	0/4	2/7	2/5	1/10	2/11	6	11	17.74
20	1/8	3/9	0/8	0/4	1/7	1/5	2/10	0/11	5	8	12.90
21	0/8	0/9	0/8	0/4	0/7	0/5	0/10	0/11	0	0	0.00
22	0/8	2/9	0/8	0/4	0/7	0/5	0/10	0/11	1	2	3.23

[a] *Acessions 1-7: diploid (2n = 30); 8: aneuploid (2n = 38); 9-15:triploid (2n = 45); 16-21: tetraploid (2n = 60); 22: hexaploid (2n = 90)*

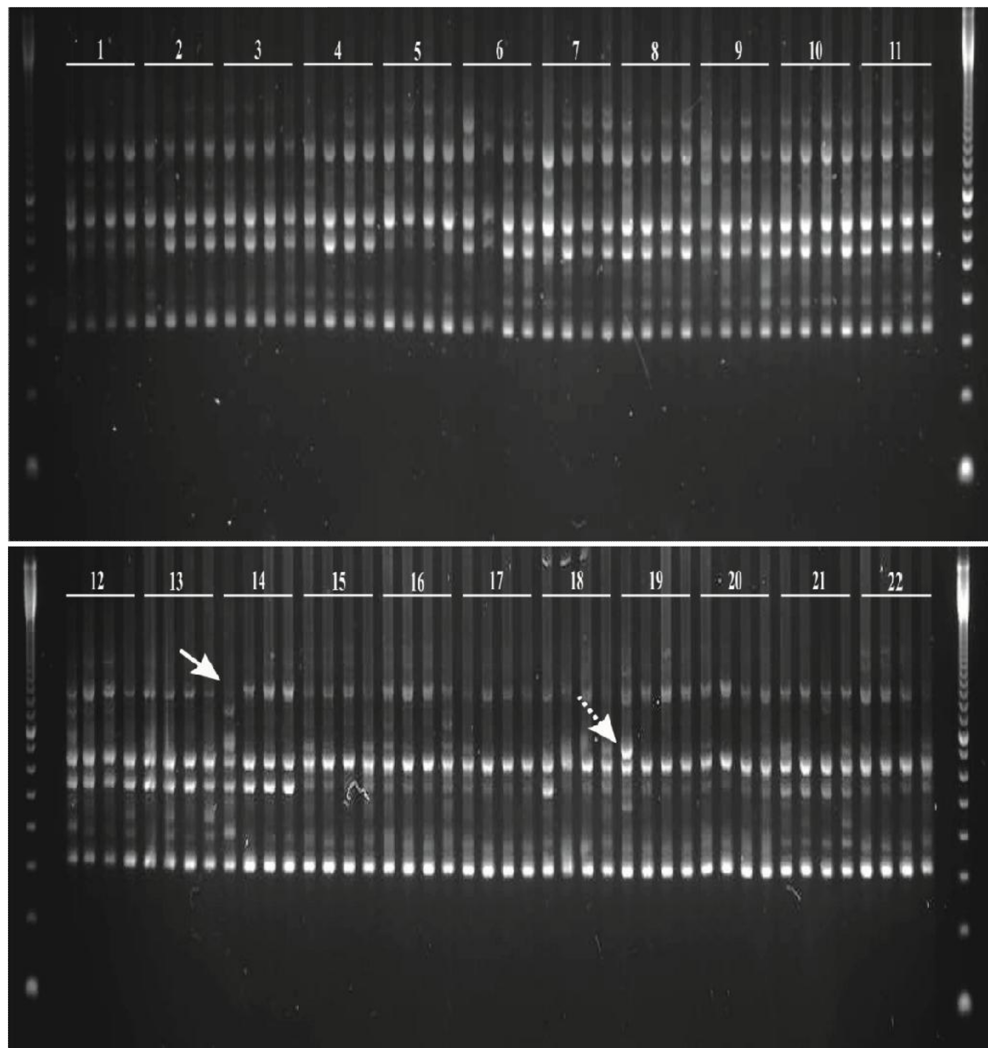

Fig. 2. Representative profile of ISSR marker in twenty two accessions of *L. alba* maintained *in vitro* and under field conditions. DNA amplification pattern was obtained with ISSR primer UBC 830. For each accession, four plants were analyzed: control plant (field conditions) followed by three replicates of *in vitro* plantlets. 100 bp ladder was used as a reference. Continuous arrow indicates example of a missing band in *in vitro* clones. Dotted arrow indicates an example of a new band in *in vitro* clones

4. DISCUSSION

In general, the *in vitro* plant regeneration procedure involved the use of growth regulators even when axillary buds were used as explants [19,20,48,49,50]. In *L. alba*, for example, the cytokinin BAP has been used in the cultivation of nodal segments with the purpose of rapid multiplication [39,40,41]. We demonstrated that *L. alba* does not require supplemental growth regulators for *in vitro* maintenance as was reported for other species such as *Bacopa monnieri* [14] and *Scutellaria baicalensis* [51]. The authors demonstrated the propagation of axillary buds on hormone-free MS medium. According to Souza, et al. [52], during nodal segment culture, the endogenous level of auxin/cytokinin might change due to the apical dominance release. The removal of the apical bud leads to an auxin level reduction, which probably favored cytokinin biosynthesis and/or inhibited its degradation at the nodal tissue, causing the development of lateral buds.

L. alba accessions retained their capacity to produce healthy roots and shoots. No changes in color, morphology or plant development were detected during seven years of *in vitro* culture. Therefore, it is clear that the species retained its growth capacity even after culturing for a long period. Usually, ploidal changes reduce the regeneration capacity of *in vitro* culture explants [53,54], which apparently was not observed in natural polyploid complex *L. alba*. All ploidal levels (diploid, aneuploid, triploid, tetraploid, and hexaploid) showed the same behavior regarding their regeneration capacity.

Flow cytometry is considered a very useful method to estimate ploidal level, DNA content, and genome size of *in vitro* cultured plant [8,55, 56]. No significant variation in the nuclear DNA content was observed in the accessions with different ploidal levels cultivated *in vitro* for seven years. Each accession was individually compared pairwise (*in vitro* vs field). Therefore, long-term culture procedures did not introduce abnormalities related to genome size in *L. alba*. Our results were consistent with those reported by Largia, et al. [14], working on *Bacopa monnieri*. They did not detect changes in nuclear DNA content and ploidy levels in plantlets maintained *in vitro* by axillary bud multiplication for 5 years as we observed for *L. alba* accessions. Flow cytometric analysis in regenerants derivated of *in vitro* somatic embryogenesis of *Smallanthus sonchifolius* also did not reveal changes of relative nuclear DNA content suggesting that the plants obtained had maintained stable its ploidal level [57]. In contrast, the increasing age of embryogenic cultures of tamarillo was cited as one of the factors responsible for expanding the abnormal ploidal level [54]. Thus, the stability of the genome under *vitro* culture may vary depending on the species and the cultivation conditions.

In addition to the estimation of DNA content and ploidal level, the genomic variations induced by tissue-cultured plants can also be detected by molecular markers [53]. The advantage of molecular markers is the possibility of detecting subtle changes in the DNA sequence [8,18,57]. The reliability and efficiency of markers in the detection of genomic instability depends on the type of marker used. The genetic variations are very likely to be reflected in the banding profiles by employing different marker systems. ISSR markers, for example, are a useful tool to detect somaclonal variation probably due to the high melting temperature of the primers that permits not only more stringent annealing but also more specific and reproducible amplification [18].

Banding profiles of *L. alba* displayed by ISSR analysis apparently showed somaclonal variation, here annotated as the presence of polymorphic bands between plants cultivated under field conditions and those ones *in vitro* cultivated. The exact cause of somaclonal variation is still unknown, although many factors have been mentioned to affect the genetic stability of *in vitro* cultures: nature of the tissue used as starting material, *in vitro* stress due to the synthetic growth environment, genotype, the number of subcultures and culture length [8]. Such factors may act together or independently to cause genetic changes in *in vitro* grown plants [58].

According to literature, the culturing of differentiated tissues such as roots, leaves, and stems, generally produces more variants than explants from axillary buds and shoot tips that have pre-existing meristems [59]. In spite of axillary bud culture being reported as the safest method to avoid genetic instability, since meristems are more stable compared to other organs [22,60,61,62], we observed a genomic instability even using axillary bud culture. *L. alba* accessions maintained *in vitro* on MS medium without growth regulators for seven years showed up to 33.87% of polymorphic ISSR bands (accession 15). In another species of the genus *Lippia* (*L. integrifolia*) the micropropagation of axillary buds without the use of growth regulators also resulted in ISSR polymorphism [63]. Similar results were reported for other species after a long-term *in vitro* culture using axillary buds [19,64], which might be explained by the loss of meristem stabilizing influence when plants are culture-grown [65]. Several factors can influence the *in vitro* cultivation, such as the culture medium, pH, temperature, cultivation time among others. Huang, et al. [19], using ISSR markers, observed 2.8% of polymorphism in long-term micropropagated plantlets of *Platanus acerifolia* after 8 years of culture on MS medium supplemented with BAP and NAA. Higher level of variation (8.75–15.63%) was detected by Dann and Wilson [64] in three potato clones subcultured by nodal cuttings for approximately 40 years on a free-MS hormone medium. In *Miscanthus×giganteus* maintained as multiple-shoot culture for 9 years on MS medium supplemented with 5.0 mg l^{-1} BAP, 8% of the

ISSR loci detected was polymorphic [66]. In contrast, no ISSR polymorphism was detected in *Bacopa monnieri* maintained *in vitro* for 5 years on MS0 medium [14] and in *Achras sapota* cultivated on medium supplemented with 2.0 mg l^{-1} of BAP for 2 years [22]. These apparently contradictory results might be explained by the differences on the genomic constitution of each species/genotype [8]. In our study, the number of polymorphic bands identified by ISSR markers ranged from 0 to 21 depending on the accession, demonstrating the genotype influence even within the same species. Such genetic instability detected by ISSR markers might be due to the occurrence of hot-spot regions within *L. alba* genome that showed more susceptibility to *in vitro* culture variation. Indeed, the existence of such regions has been suggested for other species such as *Secale cereale* [67,68], *Eucalyptus globulus* [69] and *Iris pseudacorus* [70].

The genomic instability may also be associated to physiological stress. Cultured plant tissue might input high levels of oxidative stress due to the disruption of normal developmental controls, which can ultimately lead to different types of aberrations at the nucleotide DNA sequence level [71], including, for example, the instability observed in microsatellite regions [72]. The genetic instability detected in our study may be attributed to an *in vitro* long-term cultivation increasing the harmful effects of oxidative stress. During 7 years of *in vitro* cultivation of *L. alba* accessions, they were subcultured every 2 months resulting in 42 passages. The wounding of the tissues during the excision of explant can trigger the damaging oxidative burst [73]. Along with other factors that induce somaclonal variation, the natural instability of *L. alba* genome already reported [28,36,38], might compose a complex scenario responsible for genetic variation detected in *L. alba*.

The accumulation of mutation during *in vitro* culture was previously associated to the increase of time of cultivation. The genetic variation among the donor mother plant and the regenerated plants of *Nepenthe khasiana* increased from the first (5.65%) to the third regeneration (10.87%) [74]. The authors attributed these results to an exposition increase of the regenerants to the tissue culture conditions that might induce somaclonal variations. Similarly, the subculture frequency increased the variation in *Tetrastigma hemsleyanum*, observing a peak of variation after 4-6 subcultures [75]. Moreover, the 72% of variations observed in banana was attributed to a large number of subcultures (10 generation times) [76].

As a synthetic growth environment, long-term *in vitro* culture might difficult the preservation of genetic integrity of the plants [77]. According to Us-Camas, et al. [78], the plants need to rapidly change their molecular regulation in order to respond fast and efficiently during cell division and growth. The high rate of proliferation during micropropagation achieved in relatively shorter periods led to an increase in the number of sub-cultures, increasing the stress conditions.

On the other hand, the *in vitro* culture of plant material can induce spontaneous variation but also may reveal a pre-existing variation [8]. The genetic variation rate detected in *L. alba* could also be a combination of the *in vitro* system along with the instability of the genome of the species. Considering that *in vitro* cultured genotypes are clones of the samples cultivated in the field, the genetic differences between *in vitro* and field plants are not a pre-existing character. It seems that the variations observed in *in vitro* clones occur more frequently than spontaneous mutations *ex vitro* [68,79]. It is likely that *L. alba* underwent higher spontaneous mutation rate *in vitro* than *in vivo*. Reis, et al. [28] reported that there is a considerable variation in genome size of *L. alba*. Five different chromosome numbers (2*n* = 30, 38, 45, 60, 90) and different DNA amounts were detected in 106 accessions. Another aspect is the natural karyotype variation observed even within the diploids. Lopes, et al. [29] identified high genetic diversity in diploid accessions based on SSR makers. In addition, a group of diploids seems to be a distinct lineage due to its unique DNA sequences and SSR markers. Chromosome measurements revealed a different karyotype formula reinforcing the variation among individuals of the same ploidal level [28,35,36]. The abnormalities observed during meiosis [28] also reinforce the instability of the *L. alba* genome. This scenario is consistent with the hypothesis that there is a particular labile portion of the genome especially susceptible to stress, showing higher rearrangement and mutation rates than other genome portions [80]. Repetitive sequences could be involved, since such sequences comprise a large part of plant genomes [81,82].

5. CONCLUSION

In summary, this is the first report of a long-time culture and the first study that evaluates the genetic integrity of micropropagated plants of *L. alba*. Our results suggested that the long-term culture *in vitro* did not induce nuclear DNA contents and ploidal changes in *L. alba*. This implies that the genome size of *L. alba* plantlets produced *in vitro* remained stable even after long-term culture (7 years) with repeated sub-culturing (every 2 months). ISSR analysis was efficient to detect the genome instability of the species at the molecular level, revealing a relatively high somaclonal variation rate. Such variation is possibly associated to the natural instability of *L. alba* genome combined with the long-time *in vitro* maintenance.

Considering the importance of the species and the useful aspects to maintain genetic resources *in vitro*, the instability of *L. alba* genome should be taken into account to preserve and therefore to use the species for medicinal or industrial proposes.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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