Micropropagation and ploidy stability of *Lippia lacunosa* Mart. & Schauer: an endangered brazilian medicinal plant

Diego Pandeló José[1], José Marcello Salabert de Campos[2], Lyderson Facio Viccini[2], Emilly Ruas Alkimim[1], Marcelo de Oliveira Santos[2]

[1] Universidade Federal do Triângulo Mineiro, Campus Universitário de Iturama, Iturama, Minas Gerais, Brasil. E-mail: diegopandelo@gmail.com, emillyalkimim@yahoo.com.br

[2] Universidade Federal de Juiz de Fora, Departamento de Biologia, Juiz de Fora, Minas Gerais, Brasil. E-mail: joseufjf@gmail.com, lyderson.viccini@gmail.com, marcelo.santos@gmail.com

ABSTRACT

Lippia lacunosa is a Brazilian savanna plant that belongs to the Verbenaceae family. It has been used in folk medicine as a treatment for different diseases. This species represents an endangered Brazilian medicinal plant, and this is the first report documenting a reliable protocol for the *in vitro* propagation and regeneration of *L. lacunosa*. Axenic explants were cultivated in MS medium containing different concentrations of naphthalene acetic acid (NAA) to induce root growth. The mean shoot length and the number of roots were highest with 0.06 mg·L^{-1} NAA. The highest number of buds in shoot regeneration was induced with 2 mg·L^{-1} 6-benzylaminopurine (BA). To obtain a long-term culture, the dwarf shoots were elongated on MS media containing 0.5 mg·L^{-1} BA alternated with MS containing 2 mg·L^{-1} BA every 40 days. In the present protocol, the long-term shoots retained the ability to root even after long periods of BA treatment. In addition, we evaluated the nuclear DNA content and ploidy levels, including the occurrence of endopolyploidy, in long-term micropropagated plant leaves using flow cytometry analysis. The plants propagated *in vitro* over several years possessed nuclear DNA contents ranging from 2.940 to 3.095 pg, and no differences in DNA content were found among *in vitro* plants or between these plants and the control (*L. lacunosa* from a greenhouse with a DNA content of 3.08 pg). The flow cytometry analysis also demonstrated that there was no polyploidization. The present study will be useful for biotechnological approaches and provides the first estimate of the nuclear DNA content of this species using flow cytometry.

Keywords: Brazilian savanna plant, *In vitro* propagation, Genomic stability, Flow cytometry, Disease treatment.

Estabilidade de micropropagação e ploidia de *Lippia lacunosa* Mart. & Schauer: uma planta medicinal brasileira ameaçada de extinção

RESUMO

Lippia lacunosa é uma planta do Cerrado brasileiro pertencente à família Verbenaceae e utilizada na medicina popular como uma forma de tratamento para diferentes doenças. É uma espécie ameaçada de extinção e este é o primeiro relato mostrando um protocolo eficiente para a propagação e regeneração *in vitro* de *L. lacunosa*. Os explantes axênicos foram cultivados em meio MS contendo diferentes concentrações de ANA (ácido naftalenoacético) para indução de raízes. A média do comprimento das brotações e o número de raízes foram maiores quando expostos a 0,06 mg.L^{-1} de ANA. O maior número de gemas para regeneração de brotos foi induzido sob 2 mg.L^{-1} de BA (6-benzilaminopurina). Para obtenção de cultura a longo prazo, os brotos foram alongados em meio MS com BA 0,5 mg.L^{-1}, alternando com MS contendo 2 mg.L^{-1} de BA a cada 40 dias. Os explantes mantiveram a capacidade de enraizar mesmo após longo período de tratamento com BA. Adicionalmente, foi analisado por citometria de fluxo o conteúdo de DNA nuclear e os níveis de ploidia, incluindo a ocorrência de endopoliploidia em folhas de plantas micropropagadas a longo prazo. Plantas micropropagadas durante anos apresentaram conteúdo de DNA nuclear variando de 2,940 a 3,095 pg, não sendo encontradas diferenças na quantidade de DNA em relação ao controle. A análise por citometria de fluxo também demonstrou que não houve indução de poliploidia. Os resultados obtidos podem ser utilizados para aplicações biotecnológicas e marcam o primeiro relato de quantificação do DNA nuclear desta espécie.

Palavras-chave: Planta do Cerrado brasileiro, Propagação *in vitro*, Estabilidade genômica, Citometria de fluxo, Tratamento de doenças.

1. Introduction

The *Lippia* genus comprises nearly 200 taxa (Salimena and Múlgura, 2015) and 160 species distributed mainly in Brazil, Mexico, Central America, Africa, Paraguay, and Argentina, in addition to a few endemic African species (Sousa et al., 2012; Troncoso, 1974). Recently, some efforts have been made to characterize Brazilian *Lippia* species using genetic, cytogenetic, and propagation approaches (Peixoto et al., 2006; Viccini et al., 2004, 2005). These studies have helped us to understand the genetic organization and evolution of *Lippia* species, and to characterize the germplasm bank for biological, pharmacological, and agricultural research. Their pharmacological properties have been shown to be associated with their secondary metabolites, specifically their essential oils (Gomide et al., 2016; Pascual et al., 2001).

The great challenge of flora exploration is to find valuable resources for new drug, chemical, and pharmaceutical product development (Canter et al. 2005). Verbenaceae is one of the most important families present in Brazilian rupestrian fields due to their wide occurrence and the medicinal properties of some of the genera. The amount and quality of the essential oils, as well as their medicinal properties, place the genus *Lippia* in an outstanding position within the Verbenaceae family (Campos et al., 2011). The importance of this genus is further demonstrated by the occurrence of some endemic and endangered species (Vieira, 1999). The genus *Lippia* includes 88 species, of which 68 are endemic to Brazil (Salimena and Múlgura, 2015).

Gomide et al. (2016) evaluated the anti-proliferative effect of essential oils from four species of *Lippia* in CT26.WT colon tumor cells by measuring the cell cycle phase distribution and miRNA expression. CT26.WT cells showed cell cycle arrest in G2/M phase after specific treatment. Folk medicine uses *Lippia lacunosa* to treat colds and bacterial infections. Recently, an anti-mycobacterial activity of this plant was confirmed (Leitão et al., 2006). These species can be found only northeast of the Serra do Espinhaço (Minas Gerais state), organized in small populations varying from 10 to 40 individuals. The plant produces a lot of inflorescences, but few of them produce viable seeds, hindering the plant's natural propagation.

Tissue culture is a useful tool in plant species propagation and often constitutes the first step in biotechnological studies, especially for elite genotypes with sexual inbreeding difficulties. Thus, micropropagation is an important tool to optimize the production of rare plants with important biological activities and with propagation difficulties. Considering that tissue culture can be a source of genetic variation (Palomino et al., 1999; Jain, 2001), we propose a safe and stable protocol for *L. lacunosa* micropropagation. We used flow cytometry to estimate the genome size and to analyze the stability of the nuclear DNA content and ploidy levels in plant tissues propagated for long periods on BA (6-benzylaminopurine). We propose that flow cytometry can be adopted as a standard procedure for the evaluation of somaclonal variation in this genus. This is the first report showing a reliable protocol for the *in vitro* propagation and regeneration of *L. lacunosa*.

2. Material and Methods

Representative individuals from populations collected in the Serra do Espinhaço were sent to The Botanical Experimental Station at the Federal University of Juiz de Fora, Minas Gerais, Brazil, where they were cultivated. The voucher specimens were identified by the researcher Fátima Salimena, registered at Herbário CESJ (UFJF) with the number 41,691.

Young shoots from a single genotype up to 2 cm long and without leaves were rinsed for 30 seconds with ethanol (70 % v/v), surface sterilized with sodium hypochlorite (0.4 % w/v) solution for 15 minutes with agitation, and rinsed six times with sterile water. The shoots were cultivated in test tubes containing MS media (Murashige and Skoog, 1962) supplemented with 30 g·L^{-1} sucrose. The pH of the medium was adjusted to 5.7 before adding 6 g·L^{-1} agar and was autoclaved at 120°C and 1.1 atm for 15 min. Cultures were maintained in 16/8 h (light/dark) regime, with 36 µmol·m^{-2}·s^{-1} light radiation provided by two fluorescent tubes at 26 ± 2°C. The percentage of successful explants was evaluated after one week. Shoots from nodal segments introduced *in vitro* were subcultivated in the same medium described above every 14 days and used in the subsequent experiments.

Multiple shoots were induced using MS medium supplemented with BA (0 mg·L^{-1}, 0.5 mg·L^{-1}, 1 mg·L^{-1}, or 2 mg·L^{-1}). Nodal segments 1 cm long were placed horizontally on medium containing a single node and each of them was cultivated in a jar containing 40 mL shooting media. After 40 days, the number of shoots and the average length of the elongated shoots (longer than 2 cm) were evaluated. The experiment consisted of a random design containing four repetitions of 15 explants each. The shoots obtained from the clusters cultured with MS supplemented with BA (2 mg·L^{-1} and 1 mg·L^{-1}) were then elongated on MS medium without BA for a period of 40 days in jars. Additionally, the cluster obtained from culture with 2 mg·L^{-1} BA was cut into small fragments and cultivated in different MS BA treatments (0 mg·L^{-1}, 0.5 mg·L^{-1}, 1 mg·L^{-1}, or 2 mg·L^{-1}) to induce *de novo* organogenesis.

For rooting evaluation, shoot tips 2 cm long containing two nodal segments were subcultivated in

the same medium (described above) with different NAA (naphthalene acetic acid) concentrations (0 mg·L^{-1}, 0.02 mg·L^{-1}, 0.04 mg·L^{-1}, 0.06 mg·L^{-1}, 0.08 mg·L^{-1}, or 0.1 mg·L^{-1}). All explants were incubated in a growing room at 25 ± 2°C for a 16 h photoperiod and with a photon flux density of 35 mmol·m^2·s^{-1} provided by cool-white fluorescent tubes. The treatments were arranged in a completely random design with four repetitions. The mean number of roots was calculated from 12 explants evaluated after 45 days. A total of 112 rooted plants were transferred to plastic vessels containing 50 mL of substrate (Plantmax^{TM}, Eucatex, São Paulo, Brazil) and covered with a plastic bag for four weeks in a growing room with 100 % humidity. The plants were then cultivated in air with 50–70 % humidity and the number of surviving plants was evaluated after two months. The surviving plants were transferred into 500 mL plastic bags containing a mixture of soil and sand (3:1) and were cultivated in a green house. This process was repeated twice.

To evaluate genomic stability, we used fresh leaves from 30 elongated shoots from independent clusters obtained from long-term cultures (five year old *in vitro* plants cultivated with alternating treatments for the induction of multiple shoots and elongation as described above). The shoots were elongated with the addition of BA (0.5 mg·L^{-1}) and rooted in MS medium supplemented with NAA (0.06 mg·L^{-1}) to evaluate their genomic stability using flow cytometry. For DNA content determination, approximately 20–30 mg of fresh leaf and the same weight of *Pisum sativum* fresh leaf (internal reference standard) were chopped up with a razor blade in 1 mL LB01 ice buffer to release the nuclei (Dolezel et al., 1997). The chopped tissue was aspirated through two layers of cheesecloth with a plastic pipette, filtered through a 50 µm nylon filter, and collected in a polystyrene tube. The nuclei suspensions were stained with 25 µL of 1 % propidium iodide (w/v) and 5 µL of RNase (20 mg·L^{-1}) was added to each sample. The samples were stored in a dark refrigerator and analyzed after 1 to 2 h. At least 10,000 nuclei were analyzed for each sample using a logarithmic scale display. The analysis was performed with a FacsCalibur cytometer (Becton Dickinson). Each flow cytometric histogram was saved using Cell Quest software and analyzed using WinMDI 2.8 software (available at *http://facs.scripps.edu/software.html*).

The nuclear DNA content in picograms (pg) of each species was estimated as follows:

$$Sample, 2CDNA = \frac{Peak\,channel\,of\,species}{Peak\,channel\,of\,P.\,sativum} 2CDNA\,content\,of\,standard\,(9.09pg)$$

To determine the best treatment for explant rooting and shooting the data were evaluated using an analysis of variance (ANOVA) followed by a Tukey test (p < 0.05). For the assessment of genomic stability, two leaves from each of the 30 samples were analyzed. The leaves of *L. lacunosa* maintained in a greenhouse were used as a control. Analysis of variance (ANOVA) was employed to analyze the variation in DNA content among individuals and between them and the control.

3. Results and Discussion

The level of explant contamination was high, at 70.6 %. The mother shoots started browning, and newly axenic shoots emerged 20 days after commencement of the culture. These shoots were transferred into treatments to induce the production of multiple shoots for use in subsequent experiments. Treatment with different concentrations of BA induced the production of different numbers of shoots (Figure 1A). Treatment with BA (2 mg·L^{-1}) produced the highest number of shoots, 43.83 per cluster. The treatments containing MS supplemented with 0 or 0.5 mg·L^{-1} BA produced elongated shoots (Figure 2A), whereas the treatments with 1 and 2 mg·L^{-1} BA produced severely dwarfed shoots (Figure 2B). After 40 days of culture, all shoots treated with MS with 0 mg·L^{-1} or MS with BA 0.5 mg·L^{-1} had elongated (Figure 2C). The clusters produced by treatment with MS with 2 mg·L^{-1} BA were cut into small fragments (0.25 cm^2) and transferred into different BA treatments to produce elongated shoots (Figure 1B) by *de novo* organogenesis (Figure 2D).

The axenic explants were cultivated on MS medium containing different concentrations of NAA to induce rooting. The highest average number of roots (8.48 per shoot) and the highest average length of shoots (8.96 cm) both occurred at 0.06 mg·L^{-1} NAA (Figures 1C and 1D, respectively). Plants without roots that were transferred to soil died, however, 91.2 % of plants that acclimatized after rooting survived (data not shown).

To estimate the nuclear genome size, the relative fluorescence intensity of PI stained nuclei isolated from *L. lacunosa* and *P. sativum* was compared. Distinct G1 (coefficient of variation [CV]=3.57) and G2/M peaks for *L. lacunosa* and a G1 peak for *P. sativum* (CV=2.98) were observed (Figure 3). The plants maintained *in vitro* long-term possessed nuclear DNA contents ranging from 2.940 to 3.095 pg. No statistical differences in DNA content were found among the *in vitro* plants or between these plants and the control (*L. lacunosa* from a greenhouse with a DNA content of 3.08 ± 0.0134).

The CV values obtained (Table 1) were lower than five, which is generally considered acceptable (Galbraith et al., 2001; Dolezel et al., 2007). In all histograms, only 2C and 4C peaks were detected for *L. lacunosa* (Figure 3). These results indicate that micropropagation of *L. lacunosa* under the conditions described did not introduce abnormalities such as mixoploidy or polyploidy.
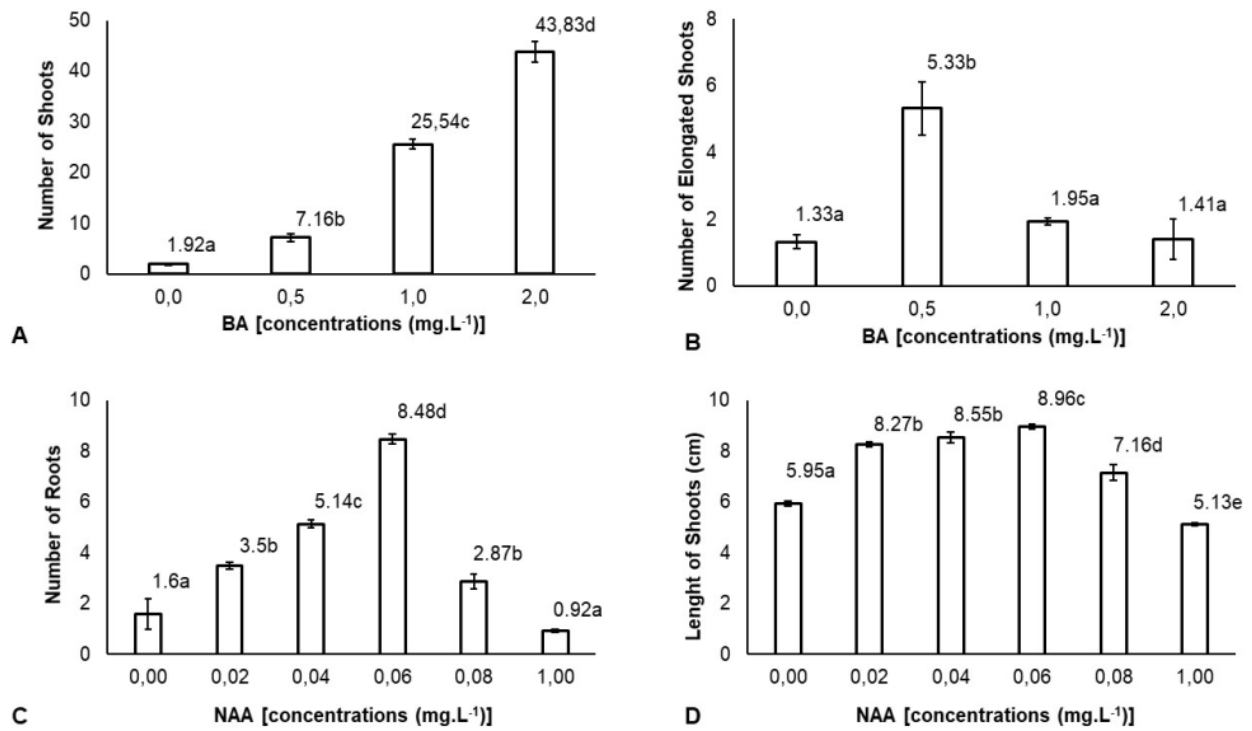
Figure 1. Number of roots (A) and length of shoots (cm) (B) in different BA concentrations. Number of shoots (C) and number of elongated shoots (D) in different NAA concentrations. Bars with the same letter are not statistically different (p < 0.05). The means are shown to the right of each bar.
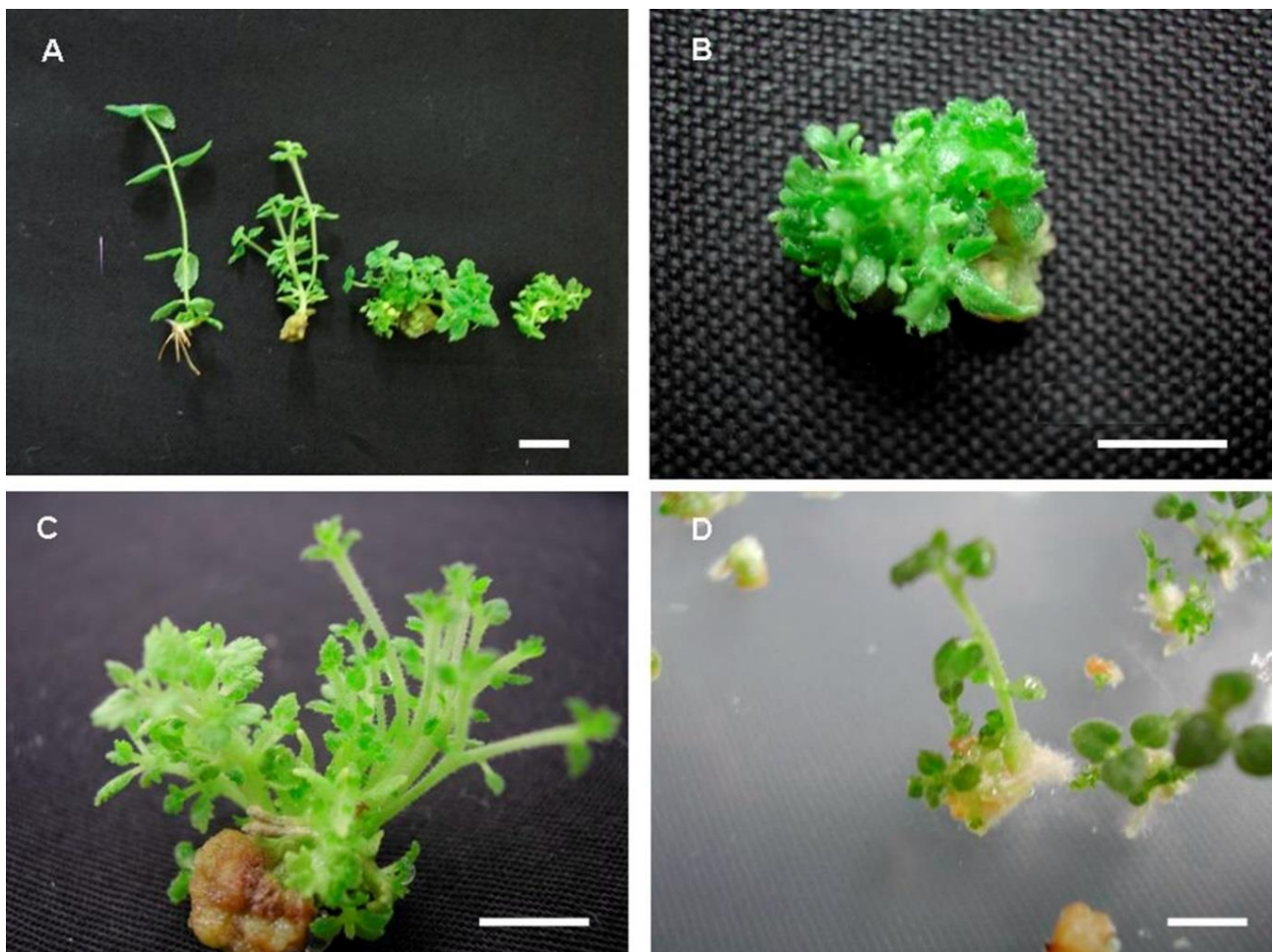

Figure 2. A) Shoots induced with BA (0, 0.5, 1, and 2 mg·L⁻¹); B) Callus induced with 2 mg·L⁻¹ BA; C) Shoots elongated long-term with 0.5 mg·L⁻¹ BA; D) Regeneration of shoots from a sliced callus (bars=1 cm).
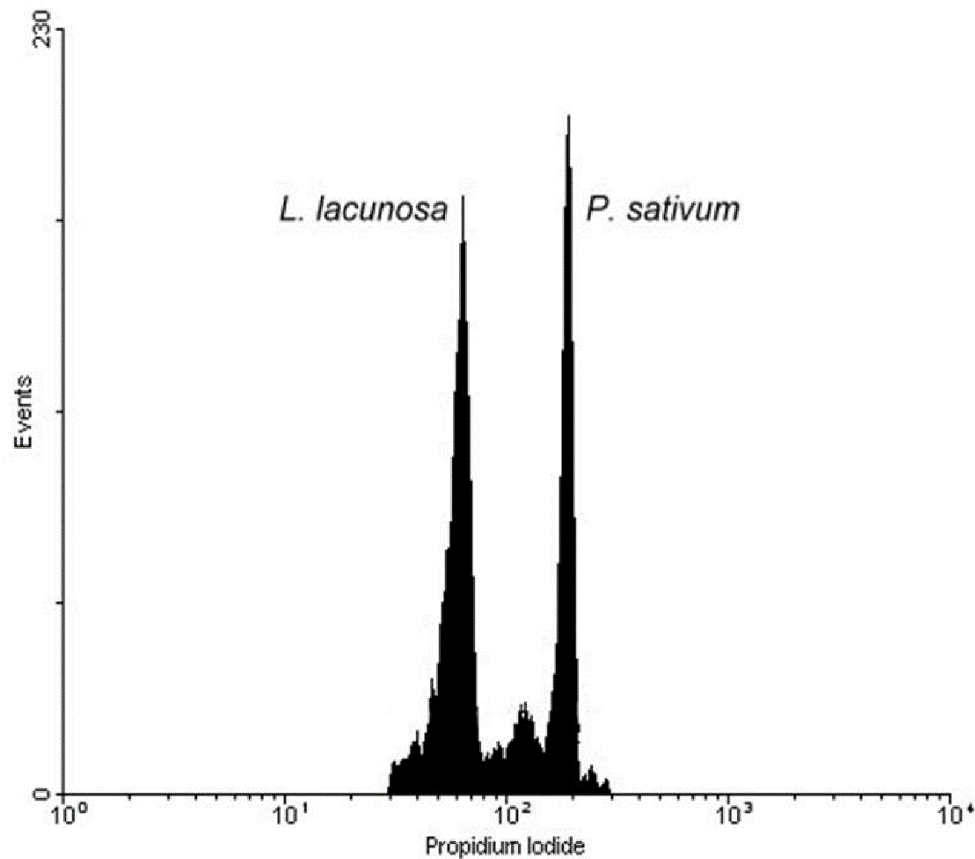
Figure 3. Histogram of DNA content estimation for *L. lacunosa* in relation to *P. sativum* (internal reference standard). Note the presence of only 2C and 4C cells for *L. lacunosa*.

Table 1. Nuclear DNA content of micropropagated plantlets and control (greenhouse *L. lacunosa*) Mean nuclear DNA content $\pm$ SD and coefficient of variation (CV) (Mean $\pm$ SD) are shown.

Plants	Nuclear DNA content (pg/2C)	CV (%)
Plantlets (individuals)	3.019 $\pm$ 0.089	4.36 $\pm$ 0.1231
Control (greenhouse *L. lacunosa*)	3.080 $\pm$ 0.013	3.43 $\pm$ 0.0234

The *in vitro* propagation method proposed in the present work produced a high percentage of stable elongated shoots after long-term BA treatment. Although the explants showed a dwarf phenotype after subculture with a dosage of 2 mg·L^{-1}, transfer of the clusters to a new medium supplemented with a low concentration of BA (0.5 mg·L^{-1}) reversed this effect and allowed them to elongate. Additionally, the sliced cluster demonstrated *de novo* organogenesis, which is useful for plant transformation. These shoots showed an optimal rooting and elongation ability under auxin treatment with NAA 0.06 mg·L^{-1}, although long periods of exposition to BA generally reduced rooting ability. These results were different from those of *Lippia filifolia,* which showed a reduced rooting ability in MS supplemented with NAA (Peixoto et al., 2006).

Thus, the sequence of treatments proposed in the present study will be useful for producing *L. lacunosa* on a large scale, and provides a valuable method for inducing multiple shoots after additional subcultures for a long period without altering the ploidy level. The long-term cultures (5 years) had been maintained in cycles of 40 days of alternating BA (2 mg·L^{-1}) and BA (0.5 mg·L^{-1}). These results indicate that this is a safe method for the propagation of *L. lacunosa*. This is an important observation, since these plantlets could be used as a source of new individuals for natural population recovery. Ploidy variations are the most common causes of genetic instability in tissue cultures, often leading to loss of the regeneration capacity of the culture material (Phillips et al., 1994).

To ensure that the long-term culture of *L. lacunosa* did not lead to somaclonal variation, we evaluated the genetic stability using flow cytometry. This tool has been extensively used for the analysis of genome stability. Loureiro et al. (2007) used this technique to analyze the ploidy stability of *Juniperus phoenicea*. In this study, the flow cytometry screening for ploidy

changes revealed no differences among plants or between them and the mother plant. The genetic stability of the medicinal plant *Scutellaria baicalensis* maintained *in vitro* has also been assessed by flow cytometry (Alan et al., 2007). No significant variation in the nuclear DNA content or ploidy levels were observed. In our report, the nuclear DNA content of *in vitro* propagated plants showed no difference compared to the control (greenhouse *L. lacunosa*). All analyses showed only 2C and 4C peaks containing an average of 85.23 % and 14.77 % of the analyzed nuclei, respectively. This pattern is considered normal for cells in an active cell cycle (Dolezel et al., 2007). Nuclei with 8C or other ploidy levels were not detected.

The importance of showing CV values in this kind of study was pointed out by Marie and Brown (1993), who suggested that CV values should be used as an elementary parameter criterion to ensure the precision of the obtained values. These authors also stated their reference guideline, a range of 1–2 % for top-quality analyses in plant cells, and around 3 % as a routine value (Marie and Brown, 1993). On the other hand, Galbraith et al. (2001) defined a CV of less than 5 % as acceptable.

Loureiro et al. (2007) reported CVs of 3.46 % for plantlets maintained *in vitro*, and Alan et al. (2007) documented CVs ranging from 3.48 % to 6.77 %. In our study, the mean CV was 4.36 % for *in vitro* plants, which was similar to other reports. However, it is still important to note, despite the fact that polyploidization was not found during the *in vitro* culture of *L. lacunosa*, the CV values obtained could mask the possible occurrence of small differences in nuclear DNA content. Consequently, the presence of a low level of aneuploidy should not be excluded.

4. Conclusions

The flow cytometry analysis excluded the occurrence of alterations such as mixoploidy and polyploidy. The development of an efficient and stable plant micropropagation procedure, and the high level of genetic stability observed in *L. lacunosa* maintained *in vitro,* provide a variety of opportunities, such as enabling long-term aseptic propagation and easy distribution of well-characterized germplasm lines of this plant species.

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