

***In vitro* propagation of *Lippia integrifolia* (Griseb.) Hier. and detection of genetic instability through ISSR markers of *in vitro*-cultured plants**

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Aromatic and medicinal plants have been traditionally harvested from the wild and, in Argentina, they have been exploited without any major limitation. *In vitro* plant propagation is an easy and inexpensive method to obtain huge amounts of plants in a short period. This practice is relevant for the propagation of valuable and endangered species, facilitating their conservation and germplasm breeding. Since tissue culture can induce genetic and phenotypic variations, collectively termed "somaclonal variation", molecular markers have been used to determine the genetic variability induced. In this work, successful propagation of *Lippia integrifolia* (Gr.) Hie ("incayuyo") was achieved with 2,2 µM of benzylaminopurine added to the Murashige-Skoog medium. Shoots were recovered from the development of axillary meristems and through *de novo* shoots regeneration from organogenic calluses. Regenerated plants were taken to an experimental trial nearby their natural habitat for their evaluation in an agricultural environment. From the moment phenotypic variants started to appear in the *ex vitro* regenerated plants, the genetic variability of both kinds of recovered materials was studied by intersimple sequence repeats. Thirteen markers were used, detecting polymorphisms with all the primers tested in both types of recovered plants. The existence of polymorphisms implies that the genetic stability must be evaluated in all the *ex vitro* recovered plants. The protocol developed here is the first step to be applied in biotechnological techniques employed to improve the quality of "incayuyo". Moreover, this is the first work employing molecular markers in *L. integrifolia*.

Key Words: Tissue culture, BAP, ISSR, Somaclonal variation

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Las plantas aromático-medicinales han sido tradicionalmente recolectadas de su medio natural, y en Argentina, han sido explotadas sin ninguna limitación. La propagación *in vitro* de plantas es un método fácil y económico para obtener grandes cantidades de plantas a corto plazo. Esta práctica es relevante para la propagación de especies valiosas y en peligro de extinción, facilitando su conservación y mejoramiento. Debido a que el cultivo de tejidos puede inducir variaciones genéticas y fenotípicas, comúnmente denominadas "variación somaclonal", los marcadores moleculares se han utilizado para determinar esta variabilidad. En este trabajo, se logró la propagación de *Lippia integrifolia* (Gr.) Hie ("incayuyo") con 2,2 µM de benzilaminopurina añadido al medio Murashige-Skoog. Los brotes fueron recuperados a partir del desarrollo de meristemas axilares y a través de la generación de brotes *de novo* regenerados a partir de callos organogénicos. Las plantas regeneradas fueron llevadas a un campo experimental, cercano a su hábitat natural, para su evaluación. Dado que se detectaron variantes fenotípicas en las plantas regeneradas, se estudió la variabilidad genética de los dos tipos de materiales recuperados a través de microsatélites anclados. Se utilizaron trece marcadores, detectándose polimorfismos con todos los iniciadores ensayados en ambos tipos de plantas recuperadas. La existencia de polimorfismos implica que la estabilidad genética debe ser evaluada en todas las plantas recuperadas. El protocolo aquí desarrollado es el primer paso para la aplicación de biotécnicas para el mejoramiento de "incayuyo". Por otra parte, este es el primer trabajo en *L. integrifolia* donde se emplean marcadores moleculares.

Palabras Clave: Cultivo de tejidos, BAP, ISSR, Variación Somaclonal

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INTRODUCTION

Several native species with aromatic and medicinal properties are commonly used in folk medicine, herbal medicine and in the flavor and food industries. In recent years, the increasing demand for these species and the extractive system used for their collection, together with the broadening of the agricultural frontier and the use of the land with urbanization purposes have caused a major decrease in wild populations leading to a loss of germplasm.

Many strategies have been employed to promote the conservation of plants, such as the raising of awareness about their value both for science and as resources for humanity (Given, 1994) and about their role in maintaining stable environments. To accomplish this goal, domestication, should be accomplished with genetic preservation and conservation is a good strategy to avoid genetic erosion (El Meskaoui, 2013). In this context, the *in vitro* plant tissue culture is a useful tool for propagation, improvement and domestication of species with economic value (Iannicelli et al., 2012a).

The genus *Lippia* comprises more than 40 species growing in Bolivia, Paraguay and in the region that stretches from the South of Brazil up to Uruguay and the Center and Northwest of Argentina (Instituto de Botánica Darwinion, 2016). *L. integrifolia* (Gris.) Hier., also known as “incayuyo” or “inca tea”, is a plant growing in the Northwest and Center of Argentina in areas close to the Andes Mountains. It is an aromatic shrub 1 m tall with grayish-brownish stems. The leaves, that are two per node, are 1-5 cm long, light green, simple, opposite and linear-lanceolate. Its flowers are about 4 mm, and originate in axillary globose compressed clusters (Zuloaga et al., 2008). In Argentina, blooming occurs at the beginning and at the end of summer.

Traditionally, the aerial parts (leaves and flowers) of “incayuyo” have been used by the local people for treatment of gastrointestinal diseases such as stomach aches and dyspepsia (Alonso & Desmarchelier 2006). Additionally, “incayuyo” has also been used as diuretic, emmenagogue and antibiotic for the treatment of cough (Ratera & Ratera 1980; Rondina et al., 2003). It is also used in the manufacturing of herbal drinks (particularly those without alcohol that are specified in the Código Alimentario Argentino (2013) also widely consumed in other South American countries. As infusion, “incayuyo” is used in a mixture with other aromatic species.

Micropropagation has been used for the rapid multiplication of many Argentinean medicinal and aromatic plants including *Lippia* species, such as *L. junelliana* (Juliani et al., 1999), *L. dulcis* (Urrea et al., 2009) and *L. integrifolia* (Passera & Ambrosetti 1999). This technique offers a way of multiplying directly and rapidly those plants possessing a special phenotypic character, and thereby shortening the time needed for the introduction of a new plant variety into the marketplace (Chaturvedi et al., 2007).

Somaclonal variation, a phenomenon associated with micropropagation, has been described by Larkin & Scowcroft (1981) as the variation arising in cell cultures, regenerated plants and their progenies. The frequencies of these variations depend on many factors like the pattern of regeneration (somatic

embryogenesis/organogenesis/axillary bud multiplication) which is one of the most important. In general, a callus phase occurrence is associated with the appearance of genetic variations (Cardone et al., 2004) while plantlets regenerated through enhanced axillary branching or direct somatic embryogenesis have been reported to be genetically uniform (Joshi & Dhawan 2007). However, the occurrence of somaclonal variations cannot be ruled out (Nookaraju & Agrawal 2012). The source of the explant, the media composition and culture conditions are other important aspects that must be taken into account as a source of genetic variation (Bairu et al., 2011).

In germplasm collection management, DNA analysis is used to study the genetic stability during *in vitro* propagation. Among the dominant multilocus DNA fingerprinting methods, the inter simple sequence repeats (ISSRs; Zietkiewicz et al., 1994) is a technique widely used to evaluate molecular markers of genetic stability (Maritano et al., 2009), to estimate genetic relationships (Pérez de la Torre et al., 2012), and to establish patterns for genomic identification (Giancola et al., 2002). The primer motif used in the ISSR markers is a microsatellite consisting of tandemly repeated short sequence motifs (1–6 bp) which are ubiquitous in eukaryotic genomes and have the potential to provide extremely polymorphic marker systems in plants (Jain et al., 1999). This technique has been successfully applied in several species (Bhatia et al., 2009; Maritano et al., 2009) including other *Lippia* species like *L. alba* (Manica-Cattani et al., 2009); however, the use of this technique to evaluate the genetic variability in *L. integrifolia* has not been reported, neither for natural populations nor for individuals regenerated under *in vitro* conditions.

This paper presents a protocol for *in vitro* propagation of incayuyo, and the use of the ISSR technique for studying the genetic variability of this species is reported. These methodologies would serve as a starting point for a breeding program aimed at achieving a sustainable profit from this genetic resource as a regional culture and also as a strategy to diminish the wild germplasm erosion.

MATERIALS AND METHODS

Plant material

L. integrifolia was collected in Dique de Olta, province of La Rioja, Argentina (30°38'25.7" S; 66°18'03.5" W; 610 m.a.s.). The botanical identification was done by our group and a voucher specimen (N° 6420) was deposited in the BAB Herbarium (Biological Resources Institute of INTA, Hurlingham, Buenos Aires, Argentina). The genotype used in this work was selected due to their chemical composition and its particular odor.

Disinfection and *in vitro* establishment of the explants

Nodal segments and apices obtained from *L. integrifolia* were used as explants. The donor plant was grown under standard greenhouse conditions.

To disinfect the explants, 50 nodal segments and apices (1 cm long) were submerged in 70% ethanol (1 min), followed by 10% commercial sodium hypochlorite (5.5%

active chlorine, 10 min) and 0.01% Tween 80. Finally, in a laminar flow cabinet, the explants were washed three times with sterile distilled water and sowed on a plant growth regulator (PGR)-free Murasigue-Skoog medium (MS) (Murashige & Skoog 1962), supplemented with 20 g l⁻¹ sucrose and solidified with 0.7% agar (Brittania™). The medium pH was adjusted to 5.6 with KOH (0.1 N and 0.5 N) and autoclaved 17 min at 121 °C and 1 atm. Each explant was grown in a 24 x 150 mm glass tube, containing 10 ml of MS medium, covered with sterile gauze/cotton plugs. After 30 days, subcultures were performed in the same medium. In all experiments, physical culture conditions consisted of a 16-h photoperiod achieved with fluorescent tubes (52 μmol.m⁻².seg⁻¹) and a temperature of 23 ± 2°C.

Culturing with bencylaminopurine (BAP)

The following concentrations of BAP were tested (μM): 0.0; 2.2; 3.3 and 4.4. A number of 15 explants were employed per treatment and the experiment was performed twice. After 45 days, multiplication rate was assessed by counting the number of shoots.

Culturing with different cytokinins

After culturing with BAP, thidiazuron (TDZ) and kinetin (KIN) were assayed in equivalent molarities to 2.2 μM BAP. The explants were obtained from the *in vitro* plantlets, as was mentioned above, in an independent experiment. The number of explants, time and culture conditions were the same as those used during the experiments employing BAP. After 45 days, multiplication rate was assessed by counting the number of shoots.

Rooting and acclimatization

Two-centimeter-long shoots from the two assays with cytokinins were transferred to the rooting medium, which was MS with a 50% dilution of the macronutrients supplemented with 0.28 μM indol-3-acetic acid (IAA). The rooted plantlets were acclimatized as described by Iannicelli et al., (2012a) by transferring them to an 8 cm diameter pot containing Growing Mix™, and maintained inside a humidity chamber. The nylon bags used to make the humidity chamber were gently perforated once per day until no inside condensation was detected. This feature determined the end of the acclimatization stage. Afterwards, plants were grown under standard greenhouse conditions.

Field Evaluation

Regenerated plants were taken to an experimental trial (Las Vizcacheras – INTA, 30°39'28" W; 66°07'15" S; 413 m.a.s) nearby Chamental, and their natural habitat (Dique de Olta, province of La Rioja), for the evaluation of growth, development and behavior.

A total of 30 plants were transplanted, spaced at a distance of 70 cm. Irrigation was started when stress or no rains were observed. After 12 months, survival percentage was obtained.

Statistical analysis

The experiments were conducted according to a complete randomized design. Multiplication rates were evaluated by analysis of variance (ANOVA) followed by

Tukey test. Significance of results was set at $P < 0.05$. The InfoStat software version 2014 (Argentina) was employed (Di Rienzo et al., 2014).

Calluses Histological Evaluation

For histological evaluation, ten calluses growing in 2.2 μM BAP were collected and fixed in FAA (formalin; alcohol; acetic acid; water 1:5:0.5:3.5) at room temperature. For inclusion in paraffin, fixed calluses were placed in glass inclusion bottles and rinsed three times with distilled water, and then dehydrated in ethanol in increasing concentrations (from 70 to 100%, 1 h each step). Clarification was then performed by successive passages in mixtures of ethanol-xylene (3:1; 1:1; 1:3, 1 h each step). Infiltration was carried out with mixtures of xylene-paraffin (3:1; 1:1; 1:3, 1 h each step) followed by a final treatment with pure paraffin. At this point, samples were placed in oven for at least 92 h at 60 °C. Serial sections (12 μm thick) were cut using a rotative Arcano microtome (model KD-1508A). Cuts were placed in slides and stretched onto a hot plate at 40 °C. Extended and dry sections were deparaffined in xylene for 2 h and then stained with Safranin-Fast green. Briefly, passages in ethanol were carried out in decreasing concentrations (100° to 96°, 2-3 min each step). A saturated solution of safranin in 80% ethanol was then added and incubated for 4 h. After incubation, samples were washed with distilled water. Samples were then stained with a saturated Fast Green solution in 100° ethanol for 30 s, followed by two rinses in ethanol 100° for 2 min and one wash with xylene for 15 min. Finally, samples were mounted in slides using artificial Canada balsam and dried until observed. Samples were observed under light microscopy (Olympus BX50FA, Tokyo, Japan), at 10X magnification.

DNA extraction

Total DNA was extracted from 30 mg young leaves of *L. integrifolia*, using liquid nitrogen and following the cetyltrimethylammonium bromide procedure according to Pérez de la Torre et al., (2010). Four plants regenerated in cultures without BAP, five plants regenerated in cultures treated with 2.2 μM BAP, and the mother plant were all subjected to DNA extraction and purification. All plants were chosen at random. Samples were incubated in the extraction buffer for 120 minutes at 65°C, after that time they were centrifuged at 13.000 rpm spin speed at 4 °C. Precipitation average volume of cold isopropanol was performed. The pellet was resuspended in 250 μL of TE 1X, and then treated with a RNase solution (1%). The obtained DNA was purified by extraction with organic solvents (phenol: chloroform: isoamyl alcohol, 25:24:1); and then, after a double aqueous extraction, the supernatant was treated with chloroform: octanol (24:1). The final precipitation was carried out with NaCl 5% and a half volume of cold isopropanol. The precipitate was washed two times with ethanol 70%.

Qualitative and quantitative measures of DNA were determined by running the samples in 0.8% agarose LE (Biodynamics®)-Tris acetate-EDTA (TAE) 1× gel, stained with ethidium bromide (0.01 mg/mL), using λ Hind III as molecular weight marker.

Evaluation of the genetic stability of the recovered plants

The genetic stability of recovered plants was assessed by ISSRs. Primers employed are listed in Table 1. The polymerase chain reaction (PCR) was carried out in a final volume of 25 μ L containing 30 ng of total DNA, 0.5 U Taq polymerase, 2.5 μ L of 10 \times reaction buffer (Kit InBio-Highway, Tandil, Argentina), 0.2 mM of each dNTP (InBio-Highway, Tandil, Argentina), 0.8 μ M primers (Operon, Alameda, CA, USA; local distributor: Tecnolab S. A., Buenos Aires, Argentina), and the corresponding concentration of MgCl₂ (InBio-Highway, Tandil, Argentina) for each primer (Table 1). DNA was amplified in a Thermal Cycler (Bio-Rad, My Cycler, CA, USA; local distributor: Tecnolab S.A.) with a preliminary step of 90 s at 94 °C, followed by 40 cycles under the following conditions: 40 s at 94 °C, 45 s at the corresponding annealing temperature for each primer (Table 1), 90 s at 72 °C, and a final 10 min extension at 72 °C. Optimal concentrations of MgCl₂ and annealing temperatures had previously been determined (Iannicelli et al., 2012b). Negative and positive controls of amplification were performed.

Ten μ L of each PCR product were analyzed in 3% agarose gels in TAE 1 \times buffer running at 80 V for 4 h. Gels were stained with ethidium bromide (0.05 mg/mL) and the MW (bp) of each band was determined by comparison with the marker (100 bp ladder, PB-L, UNQ, Quilmes, Argentina).

In order to assess the reproducibility of band profiles, DNA isolation and PCR reactions were carried out twice. Only well-defined and reproducible bands were scored. Bands displaying the same electrophoretic mobility were considered homologous fragments, regardless of their intensity.

Data analysis

The analysis was carried out with the Gel-Pro Analyzer software version 3.1. Each amplification fragment was considered an independent locus. The presence of a band was scored as "1", while the absence of the band was scored as "0". Polymorphisms were calculated using the GenAEx software version 6.5 (Peakall & Smouse, 2012).

RESULTS

Disinfection and *in vitro* establishment

After the disinfection protocol, 80% of the treated explants (nodal segments and apex) were recovered; those which were affected by ethanol and chlorine presented a brownish color due to oxidation processes. The plantlets obtained *in vitro* developed and grew normally and rooted spontaneously (Figure 1a), and they were the source of the explants used in the rest of the experiments.

Assays with BAP and other cytokinins

After treatment with BAP, callus induction at the base of the explant was observed followed by the development of shoots. After ten days of culture, the explants showed a thickening at the base (Figure 1b). After 30 days of culture, *de novo* buds developed from this morphogenetic callus (Figure 1c). As no significant differences were observed between the different treatments with BAP (Table 2), the lowest concentration of the cytokinin (2.2 μ M) was employed in the subsequent experiments, obtaining a mean value of 16 buds per explant after 45 days of culture (Figures 1d and 1e). It is noteworthy that some explants were able to produce up to 30 shoots (Figure 1e). In contrast, no development of *de novo* shoots was detected in the control explants which only presented internode elongation and adventitious root development. In all experiments no differences were found among both types of explants used (nodal segments and apex)

Table 3 shows the multiplication rates obtained with TDZ, KIN and BAP. KIN and TDZ presented no significant differences as regards multiplication rates whereas with BAP, the multiplication rate was significantly higher than TDZ and KIN.

Rooting and acclimatization

Once the shoots were 2 cm long, they were transferred to the rooting medium, where 80% of the treated plantlets developed adventitious roots (Figure 1f). These plantlets were then subjected to acclimatization procedures (Figure 1g). Finally, rooted and acclimatized plantlets were transferred and grown under greenhouse conditions. All plants were viable (Figure 1h).

Table 1. Codes and sequences of the thirteen primers (ISSRs). T: Annealing temperature. *This reference corresponds to the original sequence (GA)9T; our primer has one more T.

| Code | Primer | Sequence (5'-3') | T (°C) | MgCl ₂ (mM) | Reference |
|------|--------|------------------|--------|------------------------|--------------------|
| A | 3'GGG | GGG(TGGGG)2TG | 60 | 2.5 | UBC |
| B | 5'GACA | TC(GACA)4 | 53 | 2.5 | Jain et al. 1999 |
| C | 3'CAC | (CAC)5GT | 57 | 1.5 | Jain et al. 1999 |
| D | 3'CAG | (CAG)5AT | 55 | 2 | Jain et al. 1999 |
| E | 3'AG | (AG)8C | 53 | 2 | UBC |
| F | 3'TG | (TG)8A | 51 | 2 | UBC |
| G | 3'AC | (AC)8G | 53 | 2 | UBC |
| H | 3'TC | (TC)8A | 51 | 2 | UBC |
| I | 3'GA | (GA)10T* | 56 | 2 | Blair et al. 1999* |
| J | 5'CA | CCCGGATCC(CA)9 | 57 | 1 | Blair et al. 1999 |
| K | 5'CT | CCGGATCC(CT)9 | 67 | 2 | Blair et al. 1999 |
| L | 5'GT | CCCGGATCC(GT)9 | 67 | 2 | Blair et al. 1999 |
| M | 5'GA | CCCGGATCC(GA)9 | 67 | 2.5 | Blair et al. 1999 |



Figure 1. In vitro progress of *L. integrifolia*. a) Nodal segment cultured on free PGR MS (adventitious roots arrowed). b) Nodal segment cultured on MS + 2.2 μ M BAP after 10 days (early callus arrowed). c) Evolution of the explant cultured on MS + 2.2 μ M BAP (de novo shoots arrowed) after 30 days. d) Proliferation of new shoots in an explant cultured on MS + 2.2 μ M BAP after 45 days. e) 28 shoots recovered from one explant cultured on MS + 2.2 μ M BAP. f) Plantlets after the rooting step. g) Acclimatization phase. h) Viable ex vitro plants growing under standard greenhouse conditions.

Table 2. Multiplication rate (average number of shoots per explant) obtained after 45 days of culture with different concentrations of BAP. Different letters indicate differences between treatments (Tukey test, $p < 0.05$). Means \pm SD.

| Treatment (μ M) | Multiplication Rate |
|----------------------|---------------------|
| 0.0 | 1.8 \pm 0.7 b |
| 2.2 | 15.6 \pm 8.2 a |
| 3.3 | 8.5 \pm 4.5 a |
| 4.4 | 14.2 \pm 9.4 a |

Table 3. Multiplication rate (average number of shoots per explant) obtained after 45 days of culture with different cytokinins in equimolar concentrations of BAP. Different letters indicate differences between treatments (Tukey test, $p < 0.05$). Means \pm SD.

| Treatment (μ M) | Multiplication Rate |
|----------------------|---------------------|
| 2.2 BAP | 15.7 \pm 8.3 a |
| 2.2 TDZ | 5.7 \pm 2.7 b |
| 2.2 KIN | 3.8 \pm 1.8 b |

Calluses Histological Evaluation

Figure 2 shows the origin of the shoots recovered in vitro. Figure 2a shows the adventitious buds

development in a sector of a compact callus, whereas Figure 2b shows an early stage of shoots development with a meristematic region developing from a group of cells in the callus surface. Figure 2c shows the meristematic cone with leaf primordial in a later developmental stage.

Once plantlets were established in pots, differences in the number of leaves per node were observed, being three to four *per* node in some plantlets (Figures 3a and 3b), when the normal number is two (Figure 3c). This phenomenon was detected not only in plants regenerated from callus cultures, but also in plants developed from axillary buds.

Field Evaluation

After 12 months, 90% of the transplanted plants survived. Plants reached an average height of 1.8 m, and they developed and flowered normally (Figures 4a and 4b). At full blooming, aerial parts were collected and dried.

Genetic Stability

The thirteen primers employed (Table 1) produced 223 bands, with an average of 17 bands per primer. The number of amplicons (223) obtained in this study was suitable to reveal somatic variations. Thus, with all the primers, a polymorphism percentage of 46.6% was obtained. The changed patterns observed with these markers included the loss of original bands, the appearance of novel bands and the presence of private

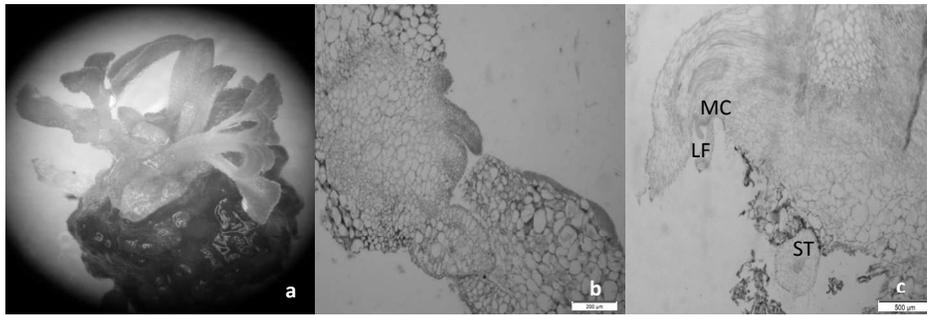


Figure 2. Organogenic response of the callus developed from the treatment 2.2 μM BAP. a) De novo shoots developed (9X). b) and c) Callus histological sections showing meristematic activity. MC: meristematic cone. LF: leaf primordia. ST: stem. Augmentation: 10X in "b" and 4X in "c".



Figure 3. Differences in the number of leaves per node of an individual from in vitro culture without BAP (a) and an individual from in vitro culture with BAP (b) and the mother plant (c).



Figure 4. *L. integrifolia* in vitro regenerated plants reintroduced in their natural habitat (Province of La Rioja, Argentina). a) Plants growing in field conditions. b) Flowering branch of an in vitro regenerated individual.

bands. Table 4 shows the percentage of polymorphism, number of loci and number of private bands obtained with each primer. The size of the amplicons ranged between 290 and 2630 bp. As an example, Figure 5 shows the amplification products profiles obtained with the primers 5'GT (Figure 5a) and 3'TC (Figure 5b). The donor of explants plant exhibited private bands with all the tested primers. These polymorphisms were detected not only between this plant and the regenerated plants through the formation of adventitious shoots from a callus via indirect organogenesis but also between the donor of explants plant and the plants developed from axillary meristems, indicating the

existence of variability between the original genotype and all the regenerated plants. Moreover, Table 4 shows that with the primer 3'TG, a polymorphism between plants derived from a callus and plants developed from axillary meristems was found. The latter phenomenon was only observed with this primer.

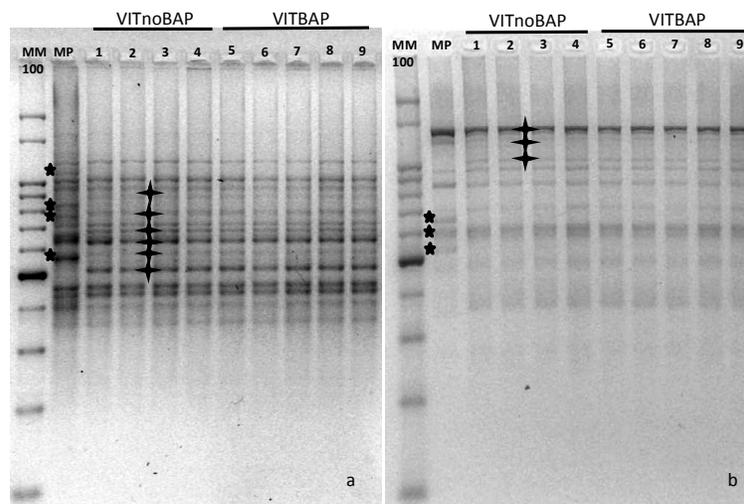
DISCUSSION

Not only does biotechnology offer the possibility of improving the production of compounds of interest, but also it gives the opportunity to overcome the problem of

Table 4. Percentage of polymorphism, number of loci and number of private bands of each primer. MP: donor of explants plant. VITnoBAP: plants recovered from in vitro culture free BAP. VITBAP: plants recovered from in vitro culture with BAP.

| Primer | % Polymorphism | Nº of loci | Nº of private bands | | |
|--------|----------------|------------|---------------------|----------|--------|
| | | | MP | VITnoBAP | VITBAP |
| 3'GGG | 38.9 | 18 | 3 | 0 | 0 |
| 5'GACA | 35.3 | 17 | 1 | 0 | 0 |
| 3'CAC | 16.7 | 6 | 1 | 0 | 0 |
| 3'CAG | 40.0 | 20 | 1 | 0 | 0 |
| 3'AG | 27.8 | 18 | 2 | 0 | 0 |
| 3'TG | 52.4 | 21 | 4 | 2 | 0 |
| 3'AC | 64.0 | 25 | 8 | 0 | 0 |
| 3'TC | 50.0 | 14 | 3 | 0 | 0 |
| 3'GA | 40.0 | 15 | 2 | 0 | 0 |
| 5'CA | 41.7 | 12 | 1 | 0 | 0 |
| 5'CT | 55.5 | 18 | 6 | 0 | 0 |
| 5'GT | 54.5 | 22 | 4 | 0 | 0 |
| 5'GA | 58.8 | 17 | 3 | 0 | 0 |

Figure 5. Band pattern obtained with the PCR amplification products using the primers 5'GT (a) and 3'TC (b). MP: donor of explants plant. The numbers indicate different individuals from: VITnoBAP: plants recovered from in vitro culture free BAP. VITBAP: plants recovered from in vitro culture with BAP. MM 100: weight molecular marker 100 bp ladder. ★ Polymorphic bands for the mother plant. ✦ Polymorphic bands for plants recovered from in vitro culture.



germplasm erosion that occurs with many medicinal and aromatic species which have become endangered due to their massive consumption and extraction from their natural habitats. Thus, it is of utmost importance to develop a protocol that allows the *in vitro* propagation of these species, which is an essential requirement for the application of biotechnological techniques to breed the species and for the massive propagation of the developed products (Iannicelli et al., 2012a).

In this study we have developed a successful method for the *in vitro* propagation of *L. integrifolia* and ISSRs markers were adjusted to determine the genetic variability of the *ex vitro* recovered plants.

As in the case of *L. integrifolia* (Passera & Ambrosetti, 1999), *L. junelliana* (Juliani et al., 1999) and *L. dulcis* (Urrea et al., 2009) the MS medium employed herein

was useful for the explants establishment.

BAP is one of the most used plant growth regulators. This cytokinin has also been used in the Verbenaceae family and different results have been obtained (Iannicelli et al., 2012a; Alderete, 2011; Miraglia, 2009; Iannicelli, 2009 and Vaccaro, 2008). In *L. integrifolia*, Passera & Ambrosetti (1999) have tried inducing callus formation with high concentrations of BAP (50 mg/L or 220 µM) but without observing any development of viable shoots under those experimental conditions. These findings might be due to the high levels of BAP employed by these authors. In *L. junelliana*, shoots formation was induced by the treatment with BAP (Juliani et al., 1999), and these authors reported a high multiplication rate obtained with 4.4 µM BAP. Taking into account these results, we decided to use similar

BAP concentrations to those employed by Juliani et al. (1999).

Since in this study no significant differences between multiplication rates obtained with 4.4 and 2.2 μM BAP treatments were found, we decided to employ the lowest cytokinin concentration in the subsequent experiments. Following a routine protocol followed in our laboratory (Iannicelli et al., 2012a; Iannicelli, 2009; Miraglia, 2009), we evaluated and compared the effect of other cytokinins in equimolar concentrations with respect to BAP. In previous studies performed with *Glandularia*, TDZ promoted the development of adventitious shoots with high multiplication rates, whereas KIN was ineffective in generating *de novo* shoots (Gonzalez Roca et al., 2015; Iannicelli et al., 2012a).

In the present work, neither KIN nor TDZ seemed to be adequate for the *in vitro* multiplication of "incayuyo", whereas BAP allowed obtaining a satisfactory multiplication rate, even at low concentrations.

From the morphological standpoint, and under the experimental conditions employed herein, a compact structure of the callus was expected since no auxin was employed; in fact, callus development was achieved only by the use of cytokinins. These results are in line with the arguments of Pierik (1990). Histologically, meristem cone formation and leaf primordia were appreciated by means of the Safranin-Fast green stain (Fuentes et al., 2005), indicating that callus had an organogenic response giving rise to *de novo* shoots. It is noteworthy that these calluses have formed in a medium supplemented only with cytokinins. However, Krikorian (1995) and Pierik (1990) have argued that the formation of shoots is possible if low concentrations of auxin and higher concentrations of cytokinins are present in the medium, with BAP being the most effective one.

Acclimatization is considered a critical phase in tissue cultures due to the occurrence of severe physiological alterations related to *in vitro* growing such as changes in leaf structure (specially the delay in the development of the cuticle and a limited functionality of the stomatal apparatus), water relations, and photosynthesis system. In this work, successful growth and development was achieved in plants after *in vitro* culture with the protocol described by Iannicelli et al., (2012a). Thus, plants could be transferred to the greenhouse conditions and then to a natural habitat in order to evaluate their growing and behavior. In this sense, rather than the collecting of plants from the wild, culture techniques have proved to be a more efficient vehicle to both preserve and exploit this source, because higher yields of biomass and essential oils accumulation can be achieved (Juliani et al., 2011).

Since the appearance of individuals with different numbers of leaves *per* node, not only in plants obtained from a callus by indirect organogenesis but also in plants developed from axillary buds of pre-existing meristems, may result in the existence of possible somaclonal variants, a molecular analysis was conducted to screen tissue culture-induced genetic variations in *L. integrifolia* plants by ISSR-PCR.

Cytological assessment has been proposed but not often used as it can be difficult in many species where chromosomes are difficult to observe. Analyses of

isozyme patterns have also been used, but they are limited in their sensitivity. It is therefore necessary to apply new approaches for studying the genetic variation in plants with related to phenotypic changes. One acceptable approach is to examine the genome of regenerants with the use of molecular markers (Nimisha et al., 2012).

ISSRs have demonstrated to be a powerful tool for the analysis of such genetic instability. By employing these markers, and working on cauliflower (*Brassica oleracea*) tissue culture Leroy et al., (2001) have detected the existence of polymorphisms. Moreover, working with *Gerbera*, Bhatia et al., (2009) have established the genetic integrity of the regenerated clones. Within the genus *Lippia*, ISSRs were successfully employed to evaluate genetic relationships and variability between different accessions of *L. alba*, finding polymorphisms among these accessions (Manica-Cattani et al., 2009), but reports about the use of these markers in the study of genetic integrity of *in vitro* cultured plants are lacking. Surprisingly, the donor of explants plant showed polymorphisms not only with respect to the regenerated plants through the formation of adventitious shoots from a callus via indirect organogenesis but also with the *ex vitro* plants developed from axillary meristems. It is known that genetic variability may occur in cultures, when plant regeneration occurs by indirect organogenesis through a callus (Cardone et al., 2004) due to a disorganized growth phase (Leroy et al., 2001; Ngezhayo et al., 2007; Rai et al., 2012). When cytokinins are added to the culture medium, the appearance of callus is expected. As one of the most used cytokinins, BAP was reported to be responsible for the formation of genetic variants (Nimisha et al., 2012; Shirani Bidabadi et al., 2010).

However, the *in vitro* propagation method used is not the only factor that determines the frequency of variations. There are other factors that might influence the occurrence of possible somaclonal variants in cultures, such as the types of tissue or the starting material, the type and concentration of plant growth regulators, the number and duration of subcultures, the genotype and the effect of stress (Bairu et al., 2011). The micropropagation technique, based in the development of axillary buds, is the most widely used system and is also considered the most suitable one to guarantee genetic stability (Bhatia et al., 2009; Joshi & Dhawan 2007). However, there exist other factors related to *in vitro* culture that cannot be eliminated and that generate stress and cause damage to the cell, such as medium definition, high salt content, water stress, and mineral deficiency. In general, alterations related to the technique itself such as oxidative damage due to wounding, cannot not be reduced either. It should be borne in mind that micropropagation entails the generation of oxidative stress (Cassells and Curry 2001) caused by the oxidizing agents employed in the sterilization of primary explants and wounded tissues in both, culture initiation and subculturing.

As it was mentioned before, Nimisha et al. (2012) and Shirani Bidabadi et al. (2010) reported the appearance of variants in cultures of *Jatropha curcas* and *Musa* spp. respectively, but they did not analyzed individuals regenerated without the adding of BAP. Instead, Devarumath et al., (2002) have reported the existence

of polymorphisms in micropropagated tea plants. These facts therefore indicate that even plants derived from axillary and organized meristems are not always genetically identical to the individuals they derive from. The reports mentioned above and our results indicate that regardless of the micropropagation technique used, a genetic stability study must be performed, since polymorphic variants were found in both types of regenerated plants. To confirm if these variants are somaclones, heritability studies should be carried out. Moreover, polymorphisms between plants derived from a callus and plants developed from axillary meristems have been detected. However, more studies with more primers, and even other molecular markers, may be necessary to confirm this finding. DNA polymorphisms detected by molecular markers have an important application as a form of DNA typing ("fingerprints") to identify different individuals, therefore the use of these markers can contribute to the identification of novel varieties. In this sense, this work is the first report of the application of molecular markers in *L. integrifolia*, and it can be useful in future development of breeding programs.

CONCLUSIONS

In the present work, an optimized *in vitro* propagation protocol for *L. integrifolia* was developed, without any apparent different nutritional conditions being required, as compared to other *Lippia* species. BAP was the cytokinin that best suited the purposes.

The ISSR markers used in this study were able to detect polymorphisms between the *in vitro* regenerated plants from both types of organogenesis and the mother plant, suggesting the existence of possible genetic variance. Thus, this work is another example that demonstrates that organized meristem cultures are not always genetically true-to-type, and not only undifferentiated tissues are a source of genetic variation. However, the heritability of these changes should be studied.

The results obtained here open an interesting scenario for the development of the germplasm toward biotechnology tools.

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