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Effect of polyploidization in the production of essential oils in *Lippia integrifolia*

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ABSTRACT

Consumption of medicinal and aromatic plants is widespread and increasing worldwide. Yet, harvesting from the wild, the main source of raw material in developing countries, is causing loss of genetic diversity and habitat destruction. This situation makes imperative the development and application of breeding programs. Autopolyploidy has brought advantages for the improvement of agronomic traits of economically important plants. In this sense, obtaining polyploid individuals is an interesting strategy to achieve this objective. In the present study successful induction of polyploidy in *Lippia integrifolia* ("incayuyo") was achieved by applying colchicine in the multiplication medium MS + 2.2 μM BAP. Induced autotetraploids showed significant differences from the field mother plant (size of the leaves, inflorescences, trichomes, stomatas, and pollen grains). In addition, essential oil yields were enhanced in tetraploids, and surprisingly, quantitative differences were detected in the composition of all recovered individuals from *in vitro* culture (tetraploids and diploids) with respect to the mother field plant. Due to the phenotypic differences, and enhanced essential oil yields and composition, tetraploid individuals became a new variety of incayuyo.

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1. Introduction

Argentina has a rich diversity of aromatic and medicinal herbs due to its varied climatic zones. One of the biggest problems facing these species is that not only are most of them collected by an extractive system, but also field use for livestock grazing, urbanization, and other human actions. These facts expose many of these species to huge genetic erosion, and virtually danger of extinction at least for several populations in the Western-Central region of Argentina (Rout et al., 2000; Cantero et al., 2005; Elechosa, 2009).

The genus *Lippia* comprises approximately 100 species of herbs, shrubs, and small trees, mainly distributed throughout South and Central America (Reis et al., 2014). Of these, more than 40 species grow in Bolivia, Paraguay and in the region that stretches from the South of Brazil up to Uruguay and the Center and Northwestern Argentina (http://www.darwin.edu.ar/Proyectos/FloraArgentina/Especies). *Lippia integrifolia* (Gris) Hier. also known as "incayuyo" or "inca tea", is a plant from the Northwest and Center of Argentina, in areas close to the Andes Mountains. It is an aromatic shrub up to 1 m tall with grayish–brownish stems. The leaves, light green color, are simple, opposite, linearlanceolate, and 1–5 cm long. White flowers, about 4 mm, born in axillary globose heads (or compressed clusters) (Zuloaga et al., 2008). It blooms at the beginning and at the end of summer.

Incayuyo is used as a mild diuretic, emenagogue, stomatic (slow digestion, bloating), and for the treatment of broncho-pulmonary diseases in popular and traditional medicine (Bassols and Gurni, 1996; Rondina et al., 2003). It is also used to develop herbal-drinks (extracts, particularly without alcohol) largely sold in Argentina and other countries in South America. As infusion, it is used in a mixture with other aromatic species.

Polyploidization has played a leading role in the evolutionary development of many plant species (Chen and Zhongfu, 2006). Chromosome doubling, known as somatic mutation, is a consequence of anomalies during mitosis.

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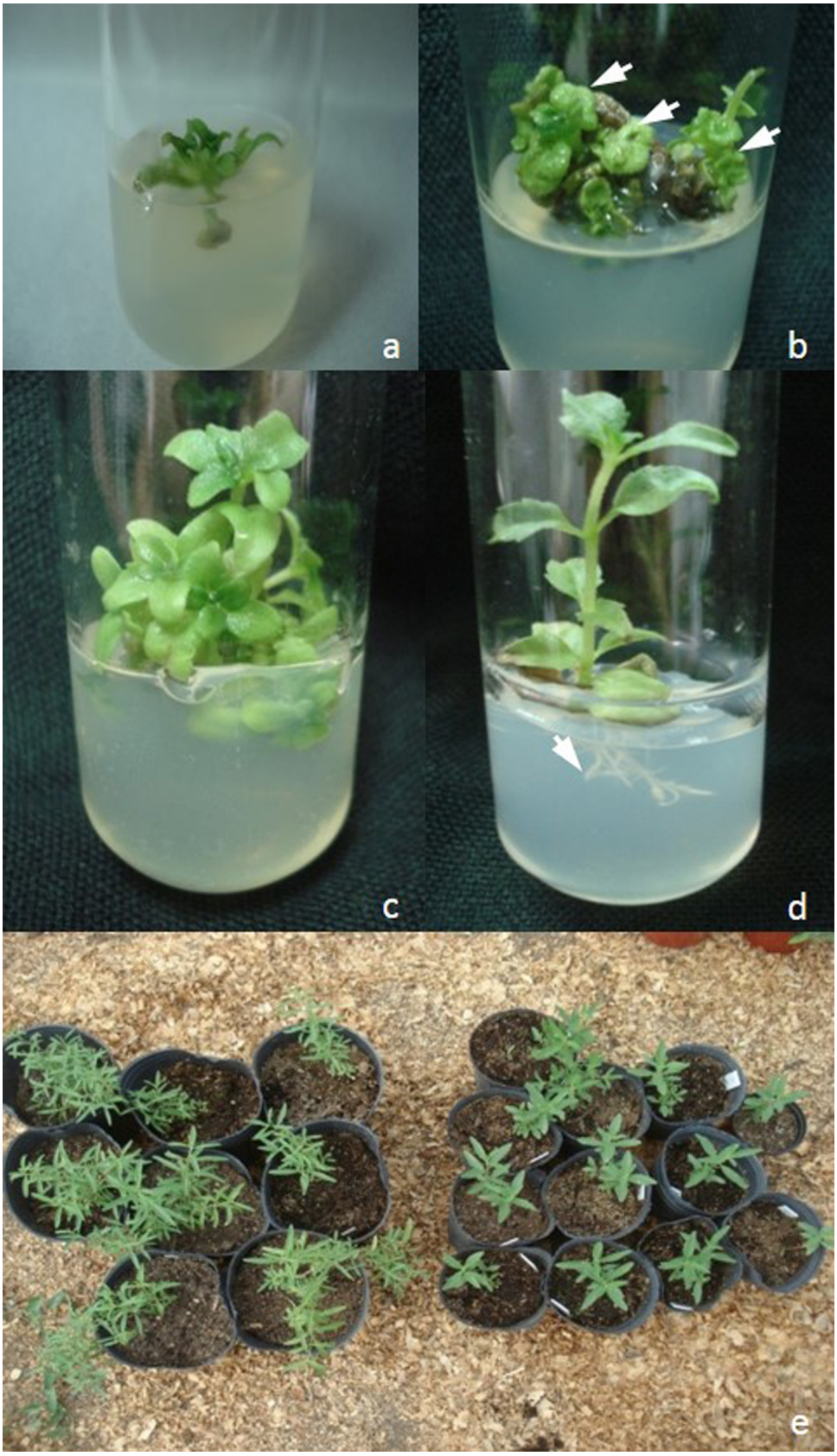

Fig. 1. *In vitro* polyploidization of L. *integridolia*. (a) Initial explant (callus induction) on MS + 2.2 μM BAP. (b) Induced shoots (arrowed) after 15 days under darkness condition, cultured on MS + 2.2 μM BAP +0.01% of colchicine. (c) Shoots proliferation on MS + 2.2 μM BAP, free of colchicine. (d) Isolated shoots during the rooting step (roots arrowed). (e) Viable *ex vitro* of diploid and tetraploid plants growing under standard greenhouse conditions (diploid plants on the left, tetraploid ones on the right).

This phenomenon is deemed very important for plant evolution, as well as, the most common mechanism that explains abrupt plant speciation. The prominence of polyploidy in flowering plants implies that it has some adaptive significance (Otto and Whitton, 2000). Polyploids often show novel phenotypes that are not present in their diploid progenitors or exceed the range of the contributing species. Some of these traits, such as increased drought tolerance, pest resistance, flowering time, organ size and biomass, could allow polyploids to enter new niches or enhance their chances of being selected for agricultural use (Osborn et al., 2003).

Plant chromosome doubling can be achieved by interfering with the cell cycle. Autopolyploids have several potential advantages since somatic doubling does not introduce any new genetic material, but rather produces additional copies of existing chromosomes. In this sense, a common effect of autoploidy is the increase of the size of both vegetative and reproductive organs of plants, often making autoploids more compact and vigorous than the corresponding diploid. Artificial induced autoploidy can be a useful and valuable tool to improve these traits within a breeding program (González Roca et al., 2015). In fact, over the last several decades, plant breeders, geneticists, and biotechnologists have been attempting to utilize ploidy level modifications, for both genetic analysis studies and applied plant production (Mishra et al., 2010; Thong-on et al., 2014). Although traditional breeding techniques, such as hybridization and mutagenesis, have played significant roles in generating desirable polyploid and haploid plants, various *in vitro* techniques have become routine in manipulating ploidy levels (Zong-Ming and Korban, 2011). In this sense, during the last decade our group have developed different new autopoliploids, such as *Scoparia* (Escandón et al., 2005), *Bacopa* (Escandón et al., 2006), *Mecardonia* (Escandón et al., 2007) and *Glandularia* (González Roca et al., 2015) autotetraploids.

Polyploids are also responsible for enhanced secondary metabolite production (Dhawan and Lavania, 1996). Recently, enhancement in secondary metabolites has been made in different medicinal and aromatic plants, such as opium (Mishra et al., 2010), *Centella* (Kaensaksiri et al., 2011), *Petunia* (Griesbach and Kamo, 1998), and *Salvia* (Gao et al., 1996) through polyploidy.

For *Lippia alba*, the possible existence of chromosomal variation suggesting the formation of a natural polyploid complex in this species was reported (Reis et al., 2014).

Except for this paper, so far no reports about the use of polyploidization in the genus *Lippia* have been found.

In order to encourage farmers to change their productive paradigm and to diminish the wild germplasm erosion, a possible strategy is to offer new varieties of aromatic and medicinal plants with added value in both price and quality, as it is expected with incayuyo.

The aim of this study was to increase the yield of essential oils of *L. integrifolia* by generating autopolyploid individuals.

2. Materials and methods

2.1. Plant material

L. integrifolia was collected at the location of Dique de Olta, province of La Rioja, Argentina (30°38′25.7″ S; 66°18′03.5″ W; 610 m.a.s.l). The botanical identification was done by our group, and the specimen was deposited in the BAB Herbarium (Biological Resources Institute of INTA, Hurlingham, Buenos Aires, Argentina, Voucher N° 6420).

2.2. In vitro *plant polyploidization*

Nodal segments of incayuyo were *in vitro* established and multiplied following the protocol described in Iannicelli et al. (2011) and Iannicelli et al. (2012b).

After 10 days on MS (Murashige and Skoog, 1962) supplemented with 2.2 μM of 6-benzylaminopurine (BAP), the explants were transferred to the same multiplication medium but supplemented with 0.01% of colchicine. The exposition time was 15 days, and the explants were cultured in darkness. The control treatments consisted of: (1) untreated nodal segments growing in the multiplication medium, and (2) nodal segments growing in the basal medium.

After colchicine treatment, the explants were transferred to the same multiplication medium for the regeneration of the new shoots. The number of explants per treatment was 25 and the assay was replicated twice.

Rooting and acclimatization were done according Iannicelli et al. (2012a). Finally, the rooted acclimatized plants were grown under standard greenhouse conditions in 8 cm pots.

Ploidy levels were determined with a flow cytometer (CyFlow Ploidy Analyser, Partec), and subsequently confirmed by chromosome counting, following the protocols described by our group in González Roca et al. (2015). The nuclear DNA of 37 colchicine treated plants and their control (20) were estimated by the fluorescence peaks obtained, and at least 10 metaphasic cells from each individual were analysed under optical microscope.

2.3. Phenotypic characterization of recovered plants

Tetraploid and diploid plants recovered from colchicine treatment, controls, and the field grown mother were characterized by measuring ten inflorescence diameters and leaves size (length and width of the third pair counting from the apex) of each group of plants. Also, the area of the trichomes, stomata (from fully developed and healthy adult leaves), and pollen grains were measured in all the groups of plants. Stomata areas were measured in fresh leaf portions. These portions were placed on slides with the abaxial side upturned and observed under microscope. Pollen grains were stained and mounted onto slides according to the protocol suggested by Alexander (1969). Both stomata and pollen grains were measured using the software Cell Sense (Copyright© 2010. Olympus Corporation), and a microscope Olympus model DP72, using an augmentation of 20X.

In all cases, the average of 10 determinations of each group of plants was taken as reference value.

Trichome areas were measured in the third leaf pair. They were observed at low vacuum, under an electronic microscope Fei model Quanta 250, using an optical gain of 500X. The same software (Cell Sense) was used, and the average of 10 determinations of each group of plants was taken as reference value.

2.4. Essential oils isolation and analysis

Essential oils of all recovered plants (control and treated plants) and the field grown mother were isolated by hydrodistillation for 2 h from 8 g of air-dried leaves using a micro-scale Clevenger-type apparatus. After cooling, settling and drying over anhydrous sodium sulphate, oils were recovered and stored at 2 °C until analysis. The yields were corrected by an average percentage of humidity. This percentage was obtained using a moisture balance Moc-1204 Shimadzu.

A total of 35 samples were quantitative and qualitative analysed by GC-FID-MS using a Perkin-Elmer Clarus 500 with a special configuration. The temperatures for the oven, injectors, detectors, transference line and the ionic source were programmed according to Retta et al. (2009). Automatic sample injection volume was set at 0.5 μl of the oil in hexane (5%), the split ratio was 80:1. A mixture of aliphatic hydrocarbons (C_6—C_{24}, Sigma–Aldrich) in hexane was co-injected using the same temperature program to calculate the linear retention index (LRI) using a generalized equation.

Table 1
Relative contents of DNA (medians) of the individuals recovered from colchicine treatments and controls. Different letters indicate significant differences (Kruskal–Wallis test, $p < 0.05$). 4X COL: tetraploid plants from colchicine treatment. 2X + 4X COL: chimera plants from colchicine treatment. 2X COL: diploid plants from colchicine treatment. 2X BAP: diploid plants from culture with only BAP, free of colchicine. 2X FBAP: diploid plants from free BAP and colchicine culture. Tetraploids are in bold.

Individuals/treatments	DAPI
4X COL	**65.23 a**
2X COL	32.21 b
2X BAP	30.51 b
2X FBAP	30.31 b
2X + 4X COL	32.26 a/59.21 b

This system configuration allowed achieving three identification parameters in a single run, LRI in both polar and nonpolar columns as well as the mass spectra of each compound.

Identification of compounds was then performed by comparison of mass spectra and retention indices obtained in both columns with those of reference compounds or those reported in the literature or with those of mass spectra libraries (Adams, 2007; Wiley, 2008). For the comparison of the oils composition, percentage composition of the essential oils components was calculated by peak area normalization (FID responses) without considering corrections for response factors. The lowest response obtained from both columns for each component was considered.

2.5. Statistical analysis

The experiments were conducted according to a complete randomized design, and all characters were evaluated during the same flowering period. Analysis of variance (ANOVA) followed by Tukey test (95%) was performed for phenotypic characterization and essential oil yields of recovered plants data. In the case of ploidy levels, Kruskall–Wallis test was carried out (95%).

The chemical composition of the essential oils of all individuals was analysed by Principal Components Analysis (PCA) using 18 variables (the main compounds of the essential oils).

In all cases, analyses were supported by the software InfoStat (Di Rienzo et al., 2014).

3. Results

3.1. Polyploidization

Although the addition of colchicine in the multiplication medium produced the oxidation of the tissues, it was possible to obtain a callus and regenerate plants when the explants were transferred to the same medium but colchicine free (Fig. 1a and b). After multiplication (Fig. 1c), shoots were isolated, rooted and transferred to greenhouse conditions (Fig. 1d and e). A total of 37 plants were recovered, of which, 13 were diploid plants, 19 tetraploid plants, and 5 chimera tetraploid plants. In contrast, no polyploidy individuals were detected in none of all the control treatments tested. It should be noted that at early growth stages, differences in the aspect of leaves were already detected (Fig. 1c). Table 1 and Fig. 2 show the differences in the relative contents of DNA of all individuals recovered.

The difference in the chromosome number between the mother plant and a tetraploid plant can be observed in Fig. 3. The chromosomes counted were $2n = 36$ for diploid genotypes (Fig. 3a) and $2n = 72$ in the colchicine treated one (Fig. 3b), thus confirming the effectiveness of induced polyploidization.

Significant differences were detected in the inflorescence diameter, leaves size, trichomes, stomata, and pollen grains area

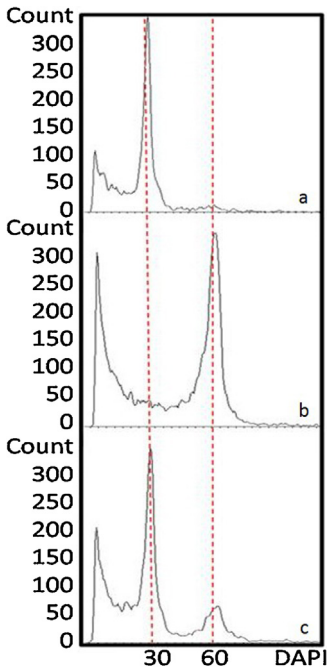

Fig. 2. Profiles of DNA content obtained by flow cytometry: (a) diploid plant; (b) tetraploid plant; (c) chimera plant.

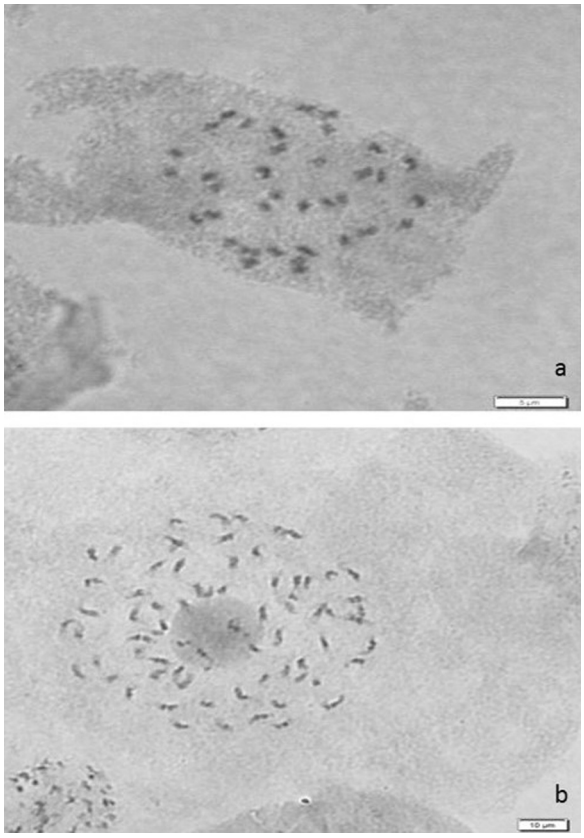

Fig. 3. Chromosome counting in L. *integrifolia*. Mitotic cells. (a) Diploid cytotypes ($2n = 2x = 36$); (b) synthetic tetraploid cytotypes ($2n = 4x = 72$). Bars = 5 μm (a) and 10 μm (b).

Table 2
Comparison of some phenotypic characteristics among the plants recovered from the colchicine assay, the controls and the mother plant. Different letters indicate significant differences (Tukey test, $p < 0.05$). FMP: field mother plant. 4X COL: tetraploid plants from colchicine treatment. 2X + 4X COL: chimera plants from colchicine treatment. 2X COL: diploid plants from colchicine treatment. 2X BAP: diploid plants from culture with only BAP, colchicine free. 2X FBAP: diploid plants from free BAP and colchicine culture. Means ± SD. n/d: not determined. Phenotypic characteristics of tetraploids plants are in bold.

Individuals/treatments	Leaves width (cm)	Leaves length (cm)	Stomatas area (μm^2)	Pollen grains area (μm^2)	Inflorescence diameter (cm)	Trichomes area (μm^2)
FMP	6.71 ± 0.86 ab	38.99 ± 3.58 a	338.55 ± 46.43 a	350.65 ± 26.14 a	0.90 ± 0.04 a	3.46 ± 0.29 a
4X COL	**11.61 ± 1.51 c**	**41.91 ± 3.79 a**	**748.09 ± 74.30 c**	**596.88 ± 35.27 b**	**3.77 ± 0.63 c**	**7.00 ± 0.51 b**
2X + 4X COL	7.66 ± 0.72 b	46.79 ± 2.64 b	536.53 ± 72.64 b	346.19 ± 21.08 a	2.81 ± 0.19 b	3.79 ± 0.54 a
2X COL	7.01 ± 0.62 ab	42.54 ± 3.01 ab	329.04 ± 35.29 a	334.25 ± 33.27 a	0.82 ± 0.07 a	3.50 ± 0.24 c
2X BAP	6.50 ± 0.53 ab	42.34 ± 2.11 ab	341.56 ± 35.93 a	347.05 ± 20.21 a	0.76 ± 0.043 a	n/d
2X FBAP	6.27 ± 0.72 a	39.00 ± 1.79 a	318.63 ± 35.02 a	330.18 ± 22.20 a	0.90 ± 0.11 a	n/d

between tetraploids and all the diploids (Table 2). However, tetraploids showed a lower trichome density than the mother plant.

Fig. 4 illustrates the differences in leaves (a), inflorescences (b), trichomes (c), stomata (d), and pollen grain (e) areas from different ploidy levels.

3.2. Essential oils composition

The essential oils isolated from all recovered individuals and the mother plant had an intense scent and yellow color.

Significant differences were detected in the essential oil yields calculated in proportion to the yield of the mother plant in tetraploids, whereas no differences were observed in diploid plants recovered (Table 3). Since very different yields were obtained in all tetraploids individuals (data not shown), Table 3 also shows the minimum and maximum yields obtained in each group. Tetraploids yielded oils in the range from 3.5–4.7%, whereas the field grown mother plant yielded 2.9%.

A total of 92 compounds were identified, representing between 90 and 94% of the total essential oil components. After analyzing 35 samples of essential oils of all type of individuals (tetraploids, diploids, both treated with colchicine and controls, and the mother plant), 18 main compounds were selected which showed contents of at least 1% as exclusion criteria. The compounds selected are listed in Table 4 in order of their elution on the DB-5 column. The studied chemotype (mother plant) was characterized by the main components β-caryophyllene (20.8%), α-humulene (11.3%), limonene (7.2%), caryophyllene oxide (8.9%), bicyclogermacrene (5.6%) and spathulenol (4.1%).

The composition of the essential oils of all individuals analyzed was qualitatively similar but showed quantitative differences. Not only did tetraploid plants, but also the diploid *ex vitro* plants, treated and not treated with colchicine, showed differences in some compounds regarding with the field grown mother plant. Most of these compounds were monoterpenes, oxygenated and not oxygenated. Although all individuals differed in their total monoterpene content respect to the mother (23.0%), particularly, tetraploids (45.2%) showed higher contents than diploids (36.2%). Sabinene was present in all the individuals in high percentages, except for the mother plant (0.8%). Although diploids displayed a higher content (8.6–5.4%), tetraploids showed the highest percentages (11.6–8.9%). Terpinen-4-ol, was also found in a high percentage (6.8–3.3% in tetraploids, 5.9–2.5% in diploids and 0.4% in the mother). The same was for gamma-terpinene, tetraploids showed a range of 1.6–2.8%, whereas diploids showed 2.1–1.0% and the mother, 0.3%. Sabinene hydrate *cis + trans* also showed higher proportions than the mother, but no huge differences were found between diploids (4.4–0.5%) and tetraploids (4.9–1.9%). A similar situation was detected for alpha-thujene and, particularly, two tetraploids were characterized by a high content of *p*-cymene (4.6–4.0%).

Chimeras showed intermediate contents, except in the case of sabinene hydrate *cis + trans*, where higher values were observed in tetraploids.

The proportions of the sesquiterpenes, also varied between the mother plant (66.7%) and the individual recovered, independently if they were diploids or tetraploids (57.2% and 47.5% respectively). Although some principal compounds like β-caryophyllene and α-humulene did not indicate major changes, some others did. Tetraploids and diploids plants were characterized by a lower content of spathulenol (3.0–1.4% and 3.2–1.8%, respectively) and caryophyllene oxide (6.1–1.9% and 7.0–2.0%, respectively) than the mother (4.1% and 8.9% respectively). Chimeras showed intermediate contents.

Three principal components (PC), which accounted for 75% of the variability, were obtained (Fig. 5). PC1, conformed by caryophyllene oxide, sphatulenol and caryophylla-4(12), 8(13)-dien-5-β-ol with the highest negative coefficients, and sabinene, α-thujene, terpinen-4-ol, α-terpineol and γ-terpinene with the highest positive coefficients, explained 34.2% of the variability and showed a clear association (cofenetic correlation = 0.98) with the type of individuals; PC2, conformed by *p*-cymene and verbenol *cis + trans* with the highest negative coefficients, and germacrene D, bicyclogermacrene, β-caryophyllene and α-humulene with the highest positive coefficients, explained 29.3% of the variability; and finally, PC3, conformed by sabinene hydrate *cis + trans* and caryophylla-4(12), 8(13)-dien-5-α-ol with the highest negative coefficients, and α-pinene and limonene, with the highest positive coefficients, explained 11.3% of the variability.

PC1 separated two large groups of samples among the individuals analyzed: those that contained the highest percentage of sabinene, and those that contained the highest of caryophyllene oxide (Fig 5). The sabinene group corresponded to the tetraploids, except for two of them, three chimeras and three diploids treated with colchicine. The group of the caryophyllene oxide included the diploids (both, treated and not treated) and one chimera. The rest two tetraploids were in a separated group. The mother plant, once field cultivated, appeared with the *ex vitro* diploid plants, but far from them, generating a new group by itself.

Table 3
Essential oils yields obtained from all the individuals recovered after colchicine treatment, the controls and the mother plant. Yields (v/w) are expressed in a range with the minimum and the maximum yield in each group. Different letters indicate significant differences (Tukey test, $p < 0.05$). FMP: field mother plant. 4X COL: tetraploid plants from colchicine treatment. 2X + 4X COL: chimera plants from colchicine treatment. 2X COL: diploid plants from colchicine treatment. 2X BAP: diploid plants from culture with only BAP, colchicine free. 2X FBAP: diploid plants from free BAP and colchicine culture. Means ± SD. Yields of tetraploids plants are in bold.

Individuals/treatments	Yields (v/w)	Yields (%) with respect to the FMP
FMP	2.9	100
4X COL	**3.5–4.7**	**135.3 ± 12.81 b**
2X COL	2.1–3.1	99.8 ± 11.1 a
2X + 4X COL	2.9–3.3	105.9 ± 6.5 a
2X BAP	2.9–3.0	102.6 ± 3.0 a
2XFBAP	2.9–3.0	101.8 ± 2.5 a

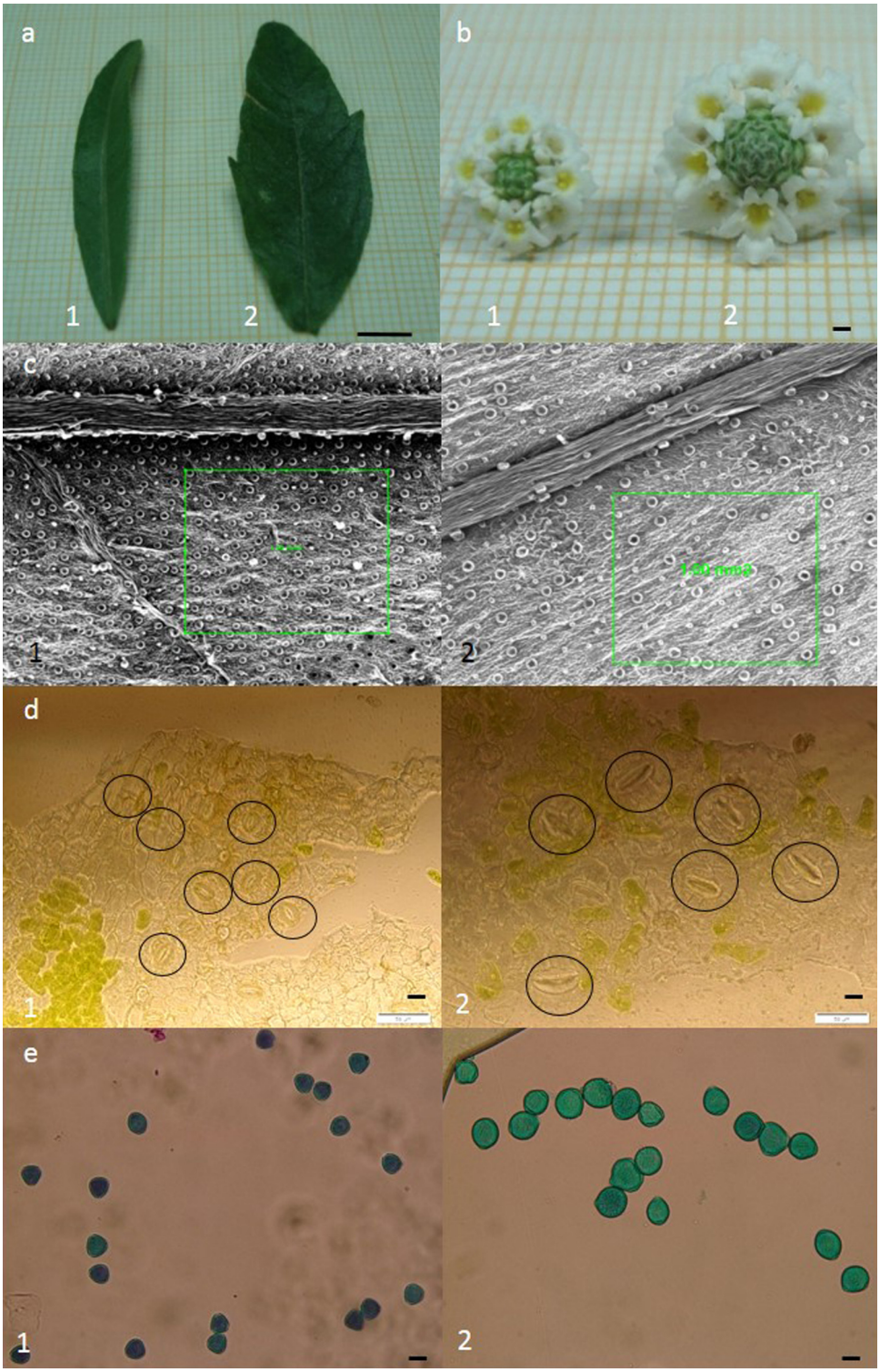

Fig. 4. Comparison of leaves, inflorescences, trichomes, stomatas, and pollen grains in diploid and tetraploid individuals. (a) (1) Leaf of a diploid individual; (2) leaf of a tetraploid plant; all leaves used as sample were taken from the 4th whorl. Barr: 5 mm. (b) (1) Inflorescence of a diploid plant; (2) inflorescence corresponding to tetraploid individual. Barr: 1 mm. (c) Trichomes of a diploid (1) and a tetraploid (2) leaf. Barr: 500 μm. (d) Stomatas of a diploid plant (1), and a tetraploid plant (2) the black circles indicate the stomata. Barr: 50 μm. (e) Pollen grains of a diploid plant (1) and a tetraploid plant (2). Barr: 50 μm.

With *p*-cymene in PC2 two tetraploids were grouped, and the rest was left in another group, and germacrene D included the diploids. The field grown mother plant, once again, appeared far from the rest diploids, in a group itself.

PC3 showed no uniform groups, but it was clearly observed that the mother plant stayed away from the remaining individuals.

4. Discussion

Biotechnology offers an opportunity to exploit the cell, tissue, organ or entire organism by growing them *in vitro*, and to genetically manipulate them to get desired compounds (Ramachandra Rao and Ravishankar, 2002). In this sense, polyploidization has

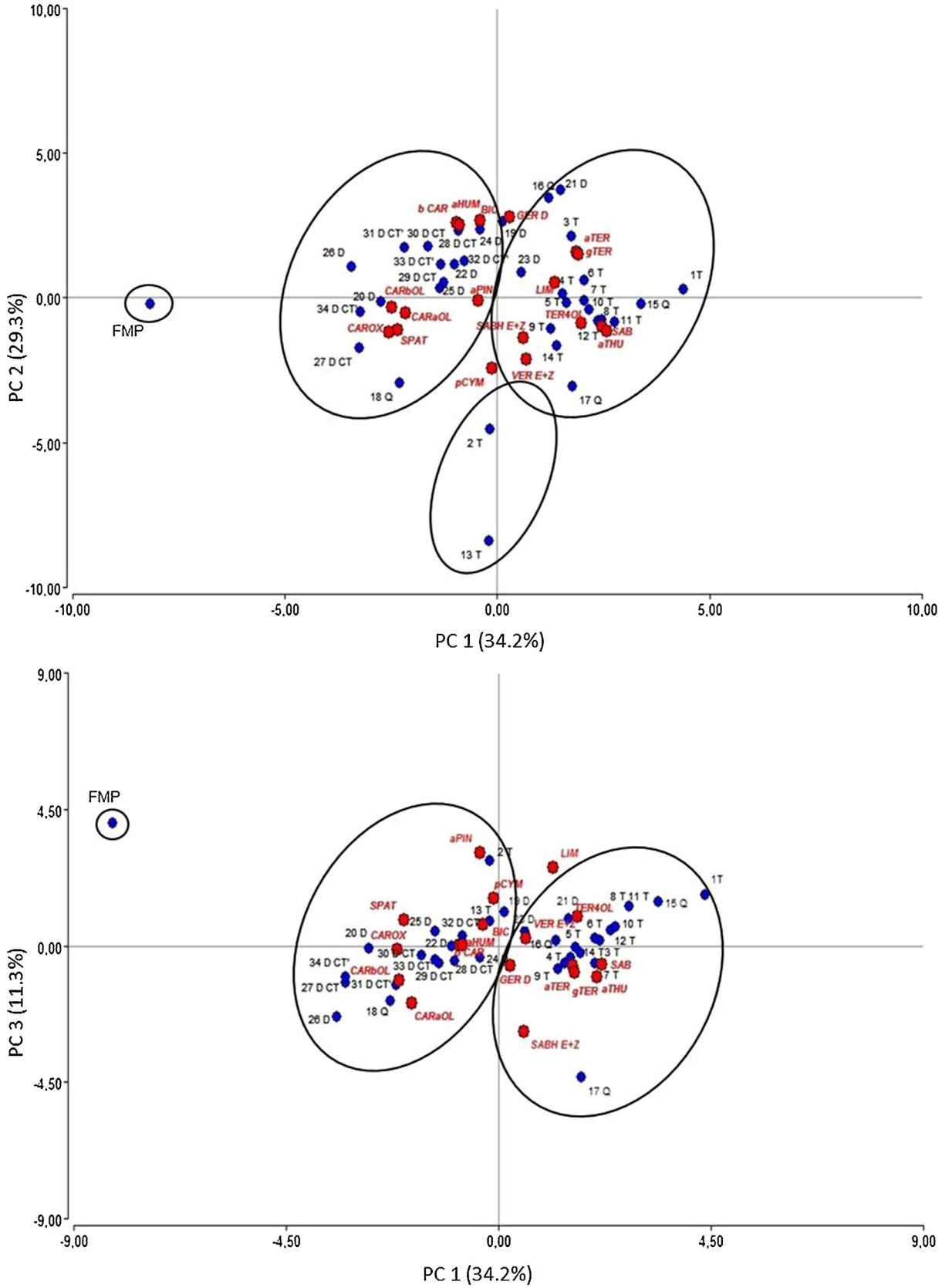

Fig. 5. Analysis of principal components of essential oils from the *in vitro* recovered individuals of L. *integrifolia* and the mother plant. FMP: field mother pant. T: tetraploids from colchicine treatment. Q: chimeras from colchicine treatment. D: diploids from colchicine treatment. D CT: diploids from culture with only BAP, colchicine free. D CT': diploids from free BAP and colchicine culture. Compound abbreviations are listed in Table 4.

Table 4
Main components (%) of the essential oils from the *in vitro* recovered individuals and the mother plant. FMP: field mother plant. 4X COL: tetraploids from colchicine treatment. 2X + 4X COL: chimeras from colchicine treatment. 2X COL: diploids from colchicine treatment. 2X BAP: diploids from culture with only BAP, colchicine free. 2X FBAP: diploids from free BAP and colchicine culture. Compounds are listed in order of elution on non-polar column and are shown in a maximum–minimum range. LRI[1]: Experimental linear retention index on non-polar column. LRI[2]: Experimental linear retention Index on polar column. *Tentatively identified.

LRI[1]	LRI[2]	Compound	FMP	4X COL	4X + 2X COL	2X COL	2X BAP	2X FBAP
945	1036	α-Thujene (aTHU)	0.2	1.8–1.4	2.0–1.3	1.4–0.9	1.3–1.2	1.3–1.0
948	1039	α-Pinene (aPIN)	3.1	2.4–1.9	1.9–1.5	2.2–1.4	2.1–1.8	2.2–1.7
985	1138	Sabinene (SAB)	0.8	11.6–8.9	11.9–7.1	8.6–5.4	7.3–0.6	7.2–6.1
1015	1206	α-Terpinene (aTER)	0.2	1.4–0.9	1.3–0.5	1.1–0.4	0.9–0.6	1.0–0.6
1017	1286	*p*-Cymene (pCYM)	1.2	4.6–0.6	1.3–0.6	1.6–0.6	1.1–0.8	1.0–0.8
1020	1223	Limonene (LIM)	7.2	8.9–5.1	9.6–4.3	7.5–5.6	6.7–1.1	6.5–5.4
1050	1266	γ-Terpinene (gTER)	0.3	2.8–0.1	2.3–1.0	2.1–1.0	1.7–1.1	1.8–1.2
1073/1100	1468/1552	cis + trans-Sabinene hydrate (IPPvsOH) (SABH E + Z)	0.5	4.9–1.9	6.4–1.6	4.4–0.5	4.1–2.2	3.4–1.7
1141/1144	1663/1685	*cis* + *trans*-Verbenol (VER E + Z)	4.5	4.9–3.6	4.7–2.9	4.1–2.3	3.9–2.8	3.9–3.3
1179	1614	Terpinen-4-ol (TER4OL)	0.4	6.8–3.3	5.0–2.9	5.9–2.5	4.2–2.8	4.7–2.6
1425	1629	*trans*-Caryophyllene (bCAR)	20.8	22.4–13.8	23.1–17.5	24.9–21.0	24.4–9.5	22.7–21.4
1453	1683	α-Humulene (aHUM)	11.3	12.2–8.1	11.3–9.4	12.3–10.3	12.2–1.0	12.3–10.3
1485	1721	Germacrene D (GER D)	0.8	1.7–0.3	1.4–0.7	1.5–1.0	1.4–1.0	1.3–1.0
1505	1775	Bicyclogermacrene (BIC)	5.6	4.5–0.6	5.5–2.0	5.7–3.7	4.8–2.4	4.5–3.1
1579	2125	Spathulenol (SPAT)	4.1	3.0–1.4	2.6–1.7	3.2–1.8	3.2–2.4	3.1–2.0
1585	1989	Caryophyllene oxide (CAROX)	8.9	6.1–1.9	6.0–2.4	5.9–2.0	7.0–0.9	6.4–4.0
1632	1940	Caryophylla-4(12), 8(13)-dien-5-α-ol* (CARaOL)	1.2	1.2–0.4	1.2–0.5	1.3–0.6	1.2–0.9	1.6–0.9
1636	1949	Caryophylla-4(12), 8(13)-dien-5-β-ol* (CARbOL)	3.4	2.4–0.6	2.8–0.8	2.8–0.9	3.2–1.8	3.3–2.0
		Total	**90.8**	**93.5**	**92.5**	**94.1**	**93.8**	**93.0**
		Monoterpenes	16.0	29.0	26.5	23.6	23.0	22.1
		Oxygenated monoterpenes	7.0	16.2	15.3	12.6	12.5	12.3
		Total monoterpenes	**23.0**	**45.2**	**41.8**	**36.2**	**35.5**	**34.4**
		Sesquiterpenes	42.2	34.8	36.0	42.5	40.8	41.2
		Oxygenated sesquiterpenes	24.5	12.7	13.9	14.6	16.7	16.7
		Total sesquiterpenes	**66.7**	**47.5**	**49.9**	**57.2**	**57.6**	**57.9**
		Others	1.1	0.9	0.9	0.7	0.7	0.7

demonstrated to be a powerful tool to increase the production of secondary metabolites.

The polyploidization procedure needs to be integrated in the *in vitro* protocols since antimitotic agents are used in media, including the start of the culture and clonal propagation phases (Dhooge et al., 2011). Based on previous experience in the *Verbenaceae* family in our work group, we tried to reproduce the protocol described by González Roca et al. (2015), in which the explants were submerged in a solution with or without the addition of colchicine for 24 and 48 h. In both exposure times, no explants survived (data not shown).

In the present study, successful induction of polyploidy was achieved through adding colchicine to the multiplication medium. Chromosome doubling often has a later effect on the proliferation, regeneration rate and shoots elongation, which are often retarded after treatment with antimitotic agents (Dhooge et al., 2011). Growth and development is often slowed in polyploid plants, leading to a delayed and prolonged flowering phenology. Also, shoots seemed to grow more slowly after the exposure to colchicine (Ramsey and Schemske, 2002; Mishra et al., 2010). This fact and the differences observed in the phenology of the leaves at very early growth stages, were the first indications of the successful colchicine treatment. In this sense, it is important to point out that the slow growth of the tetraploid plants was only observed during the *in vitro* culture and acclimatization phases, and when the plantlets were carried to the standard greenhouse they grew and flowered equally well as the wild type. Thong-on et al. (2014) working with synthetic autopolyploids of *Centella* reported an optimal growth of these polyploids when they were evaluated in field conditions.

Colchicine caused an increase in the mortality rate of the explants and plantlets *in vitro*. This fact has been already reported by other authors (Kadota and Niimi, 2002; Kaensaksiri et al., 2011), possibly due to physiological disturbances that could have resulted in a reduction of the cell division rate (Yemet and Blume, 2008).

Polyploids are commonly differentiated from parental diploids by a combination of morphological, reproductive, phenological, life-history and physiological traits, such as increased cell and organ sizes, and sometimes greater vigor and biomass, and new phenotypic and molecular variations can arise shortly after polyploid formation (Ramsey and Schemske, 2002). In this work, differences in the size of leaves, inflorescence, trichomes, stomatas and pollen grains were found between the new tetraploids and the diploids. In other medicinal and aromatic plants, especially the stomata size was used for the identification of polyploids (Rauf et al., 2006; Mishra et al., 2010; Kaensaksiri et al., 2011).

All these differences are in agreement with previous works in poliploidization from our group (Escandón et al., 2005, 2006, 2007; González Roca et al., 2015), who reported an increase in all these traits in induced tetraploid plants. Since cells with a larger complement of chromosomes grow larger to maintain a constant ratio of cytoplasmic to nuclear volume, and express more proteins with the presence of more genes, consequently, an increase in the organ size is expected (Rauf et al., 2006).

Because essential oils are produced and stored in trichomes, we measured the size of these structures in several individuals. Trichomes of tetraploid plants were larger than those in the diploids, and this may be related to the increase in the essential oil yields observed in these individuals.

All the individuals recovered from the control treatments were diploid, and some that had been treated with colchicine were also diploid. It is important to highlight that from the phenotypic point of view, no differences were detected between the diploid individuals recovered, either from the control or the colchicine treatments.

For medicinal and aromatic plants, polyploids are usually more valuable because they exhibit increased biomass and content of effective compounds (Gao et al., 1996). Since the autopolyploids arise as a consequence to direct genomic multiplication, the basic genetic material remains the same with multiplied gene dosage. Therefore, an enhanced production of metabolites becomes imperative in the autotetraploids (Dhawan and Lavania, 1996).

In this work, tetraploids exhibited higher essential oil yields than diploids and the field mother plant, showing an average

increase of 35%, some of them yielded almost 60% more (data not shown). This effect had been reported earlier in several aromatic species, like *Carum* (Zderkiewicz, 1971; Dijkstra and Speckmann, 1980), *Lavandula* (Rabotyagov and Akimov, 1990) and *Ocimum* (Bose and Chaudhury, 1962).

It is significant to note that this increase in the essential oil yields was accompanied with the fact that tetraploids exhibited larger leaves and trichomes size than diploids, and although further studies are necessary, as a first approximation, this fact is a possible explanation for the increase in the production of essential oils.

No qualitative changes were observed in the composition of the essential oils, whereas quantitative changes were detected in the composition of all *ex vitro* material, regardless of ploidy level, compared with the composition of the mother plant. This fact is confirmed by the differences found on the total content of monoterpenes and sesquiterpenes between all *ex vitro* individuals and the mother plant. These differences may have occurred because under *in vitro* conditions, young shoots are produced and the metabolites that are secreted by trichomes are accumulated and concentrated in shoots (Sudriá et al., 1999; Arikat et al., 2004; Zielinska et al., 2011).

Tetraploid plants showed the highest levels of monoterpenes. Quantitative differences in secondary metabolites following polyploidy induction have been reported earlier in the developed tetraploids of several plants like *Salvia, Petunia, Artemisia, Papaver, Centella,* and others, in which secondary metabolite accumulation was enhanced in comparison to their diploid counterpart (Gao et al., 1996; Griesbach and Kamo, 1998; De Jesus-Gonzalez and Weathers, 2003; Mishra et al., 2010; Kaensaksiri et al., 2011 respectively).

As it was mentioned before, tetraploids monoterpenes were the most affected compounds, and this fact allowed separating the different groups in the PCA (tetraploids, diploids from colchicine and controls, and the mother plant alone). Sesquiterpenes showed no significant changes, except for some compounds. Our results suggest that the metabolism of the monoterpenes may be more affected by the polyploidization than the sesquiterpenes. Thus, the enzyme activity of different monoterpene synthase might be increased after polyploidization.

Although somatic chromosome doubling does not introduce new genetic material and produces only additional copies of existing genes and chromosomes, many genome alterations occur after mitotic polyploidization (Ranney, 2006). In this sense, the appearance of novel phenotypes in new polyploid plants probably involves changes in gene expression. Examples are loss of duplicated genes, gene expression alterations and epigenetic changes modulating gene expression (Osborn et al., 2003; Adams and Wendel, 2005; Parisod et al., 2010; Yang et al., 2011). These genetic changes often result in polyploid crops being superior to diploids with respect to morphological changes, genetic adaptability and tolerance to environmental stresses, and, the most important feature in aromatic plants, biomass and content of bioactive compounds increments (Xiong et al., 2006). The exact reason for these differences is still unclear, but it might be due to the advantage of polysomic inheritance. This could result in an increased genome flexibility that might allow a better response to environmental changes (Parisod et al., 2010). Moreover, polyploid individuals have an overall increased gene expression level compared to diploids, which could result in an affected dosage-regulated expression (Osborn et al., 2003).

Using the protocol presented in this paper, an incayuyo with enhanced essential oils production and larger leaves and inflorescence was obtained. Because these phenotypic characteristics remained more than 12 months after obtaining the new genotypes, they were presented as a new variety of incayuyo, allowing their incorporation, possibly, into a *L. integrifolia* breeding program.

5. Conclusions

In the present work, an optimized *in vitro* polyploidization protocol for *L. integrifolia* is presented. The obtaining of incayuyo autotetraploids can be possible through adding colchicine to the multiplication medium. The duplication of genetic information was correlated with the duplication of the traits studied in this species, especially in the production of secondary metabolites. Tetraploid plants are a valuable and promising material as a new variety of incayuyo.

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References

Adams, R.P., 2007. Identification of Essential Oils Components by Gas Chromatography/Quadrupole Mass Spectroscopy. Allured Publ. Corp., Carol Stream, IL.

Adams, K.L., Wendel, J.F., 2005. Novel patterns of gene expression in polyploid plants. Trends Gen. 21 (10), 539–543.

Alexander, M.P., 1969. Differential staining of aborted and non-aborted pollen. Stain Technol. 44, 117–121.

Arikat, N.A., Jawad, F.M., Karama, N.S., Shibli, R.A., 2004. Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa* Mill). Sci. Hortic. 100, 193–202.

Bassols, G., Gurni, A., 1996. Especies del género *Lippia* utilizadas en medicina popular latinoamericana. Dominguezia 13, 7–25.

Bose, R.B., Chaudhury, J.K., 1962. A comparative study of the cytotaxonomy, palynology, physiology of diploid plants from *Ocimum kilimanscharicum* Guerke and their yield of raw material and volatile contents. Caryologia 15, 435–454.

Cantero, P., Thomas, H., Ernst, E., 2005. Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. Trends Biotechol. 23 (4), 180–185.

Chen, Z., Zhongfu, N., 2006. Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. Bioessays 28, 240–252.

De Jesus-Gonzalez, L., Weathers, P.J., 2003. Tetraploid *Artemisia annua* hairy roots produce more artemisinin than diploids. Plant Cell Rep. 21, 809–813.

Dhawan, O.P., Lavania, U.C., 1996. Enhancing the productivity of secondary metabolites via induced polyploidy: a review. Euphytica 87, 81–89.

Dhooge, E., Van Laere, K., Eeckhaut, T., Leus, L., Van Huylenbroeck, J., 2011. Mitotic chromosome doubling of plant tissue *in vitro*. PCTOC 104, 359–373.

Dijkstra, H., Speckmann, G.J., 1980. Autotetraploidy in caraway (*Carum carvi* L.) for the increase of the aetheric oil content of the seed. Euphytica 29, 89–96.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, C.W., InfoStat versión, 2014. Grupo InfoStat, FCA. Universidad Nacional de Córdoba, Argentina http://www.infostat.com.ar.

Elechosa, M.A., 2009. In: INTA (Ed.), Manual de Recolección Sustentable de Plantas Aromáticas Nativas de la Región Central y Noroeste de la Argentina. Proyecto Específico PNHFA4164, Buenos Aires, p. 48.

Escandón, A.S., Miyajima, I., Alderete, L.M., Hagiwara, J.C., Facciuto, G., Mata, D., Soto, M.S., 2005. Wild ornamental germplasm exploration and domestication based on biotechnological approaches, *in vitro* colchicine treatment to obtain a new cultivar of *Scoparia montevidiensis*. Electron. J. Biotechnol. 8, 204–211.

Escandón, A.S., Hagiwara, J.C., Alderete, L.M., 2006. A new variety of *Bacopa monnieri* obtained by *in vitro* polyploidization. Electron. J. Biotechnol. 9, 157–162.

Escandón, A.S., Alderete, L.M., Hagiwara, J.C., 2007. A new variety of *Mecardonia tenella*, a native plant from South America with ornamental potential, obtained by *in vitro* polyploidization. Sci. Hortic. 115, 56–61.

Gao, S.L., Zhu, D.N., Cai, Z.H., Xu, D.R., 1996. Autotetraploid plants from colchicine-treated bud culture of *Salvia miltiorrhiza* Bge. PCTOC 47, 73–77.

González Roca, L., Iannicelli, J., Coviella, A., Bugallo, V., Bologna, P., Pitta Álvarez, S., Escandón, A., 2015. A protocol for the i*n vitro* propagation and polyploidization of an interspecific hybrid of *Glandularia* (*G. peruviana x G. scrobiculata*). Sci. Hortic. 184, 46–54.

Griesbach, R.J., Kamo, K.K., 1998. The effect of induced polyploidy on the flavonol of *Petunia* 'Mitchell'. Growth Metab. 42, 361–363.

Iannicelli, J., Gonzalez Roca, L., Irigoyen, E., Elechosa, M.A., Escandón, A.S., 2011. Macro y micropropagación *in vitro* de Lippia integrifolia (incayuyo) y L. junelliana (salvialora): especies aromáticas de importancia industrial. In: VIII Simposio Nacional de Biotecnología REDBIO Argentina, CABA, Argentina.
Iannicelli, J., Miraglia, M.C., Alderete, L.M., Pitta Álvarez, S., Escandón, A.S., 2012a. *In vitro* propagation of *Glandularia peruviana* (L.) Small, an ornamental native plant from South America. Rev. Fca UnCuyo. 44 (2), 119–130.
Iannicelli, J., Gonzalez Roca, L., Elechosa, M.A., Escandón, A.S., 2012b. Avances en la multiplicación *in vitro* de *Lippia integrifolia* (incayuyo). Dominguezia 28 (2), 55–56.
Kadota, M., Niimi, Y., 2002. *In vitro* induction of tetraploid plants from a diploid Japanese pear cultivar (*Pyrus pyrifolia* N. cv. Hosui). Plant Cell Rep. 21, 282–286.
Kaensaksiri, T., Soontornchainaksaeng, P., Soonthornchareonnon, N., Prathanturarug, S., 2011. *In vitro* induction of polyploidy in *Centella asiatica* (L.) urban. PCTOC 107, 187–194.
Mishra, B.K., Pathak, S., Sharma, A., Trivedi, P.K., Shukla, S., 2010. Modulated gene expression in newly synthesized auto-tetraploid of *Papaver somniferum* L. South Afr. J. Bot. 76, 447–452.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 437–497.
Osborn, T.C., Pires, J.C., Birchler, J.A., Auger, D.L., Chen, Z.J., 2003. Understanding mechanisms of novel gene expression in polyploids. Trends Gen. 19 (3), 141–147.
Otto, S., Whitton, J., 2000. Polyploid incidence and evolution. Annu. Rev. Genet. 34, 401–437.
Parisod, C., Holderegger, R., Brochmann, C., 2010. Evolutionary consequences of autopolyploidy. New Phytol. 186, 5–17.
Rabotyagov, V.D., Akimov, A.Yu., 1990. Inheritance of essential oil content and composition in tetraploids and sesquidiploids of lavender. Genetika (Mosk.) 26, 283–292.
Ramachandra Rao, S., Ravishankar, G.A., 2002. Plant cell cultures: chemical factories of secondary metabolites. Biotechnol. Adv. 20, 101–153.
Ramsey, J., Schemske, D.W., 2002. Neopolyploidy in flowering plants. Annu. Rev. Ecol. Syst. 33, 589–639.
Ranney, T.G., 2006. Polyploidy: from evolution to new plant development. Proc. Int. Plant Propag. Soc. 56, 137–142.
Rauf, S., Khan, I.A., Khan, F.A., 2006. Colchicine-induced tetraploidy and changes in allele frequencies in colchicine-treated populations of diploids assessed with RADP markers in *Gossypium arboretum* L. Turk. J. Biol 30, 93–100.
Reis, A.C., Sousa, S.M., Vale, A.A., Pierre, P.M., Franco, A.L., Campos, J.M., Vieira, R.F., Viccini, L.F., 2014. *Lippia alba* (Verbenaceae): a new tropical autopolyploid complex? Am. J. Bot. 101, 1002–1012.
Retta, D., Gattuso, M., Gattuso, S., Di Leo Lira, P., van Baren, C., Ferraro, G., Bandoni, A., 2009. Essential oils composition of *Achyrocline flaccida* (Weinm.) DC. (Asteraceae) from different locations of Argentina. Biochem. Syst. Ecol. 36 (12), 877–881.
Rondina, R., Bandoni, A., Coussio, J., 2003. Plantas Silvestres Argentinas con Reconocidas Propiedades Medicinales o Tóxicas. OEA-CYTED, CD-ROM, Buenos Aires.
Rout, G.R., Samantaray, S., Dasa, P., 2000. *In vitro* manipulation and propagation of medicinal plants. Biotech. Adv. 18, 91–120.
Sudriá, C., Piñol, M.T., Palazón, J., Cusidó, R.M., Vila, R., Morales, C., Bonfill, M., Cañigueral, S., 1999. Influence of plant growth regulators on the growth and essential oil content of cultured *Lavandula dentata* plantlets. PCTOC 58, 177–184.
Thong-on, W., Arimatsu, P., Pitiporn, S., Soonthornchareonnon, N., Prathanturarug, S., 2014. Field evaluation of in vitro-induced tetraploid and diploid *Centella asiatica* (L.) urban. J. Nat. Med. 68, 267–273.
Wiley/NIST, 2008. The Wiley/NBS registry of mass spectral data. 8th Ed. J. Wiley & Sons, Inc., New York/NIST/EPA/NIH (2005) Mass Spectral Library, vers. 2.0.
Xiong, Y.C., Li, F.M., Zhang, T., 2006. Performance of wheat crops with different chromosome ploidy: root-sourced signals, drought tolerance, and yield performance. Planta 224, 710–718.
Yang, C., Zong-Ming, C., Tschaplinski, T.J., Wullschleger, S.D., Weilun, Y., Xinli, X., Tuskan, G., 2011. Genomic aspects of research involving polyploid plants. PCTOC 104, 387–397.
Yemet, A.I., Blume, Y.B., 2008. Progress in pant polyploidization based on antimicrotubular drugs. Open Hortic. J. 1, 15–20.
Zderkiewicz, T., 1971. Content of oil in different stages of ripe fruits of diploid and tetraploid cumin (*Carum carvi* L.). Acta Agrobot. 24, 121–127.
Zielinska, S., Piatczak, E., Kalemba, D., Matkowski, A., 2011. Influence of plant growth regulators on volatiles produced by *in vitro* grown shoots of *Agastache rugosa* (Fischer & C.A. Meyer) O. Kuntze. PCTOC 107, 161–167.
Zong-Ming, C., Korban, S.S., 2011. *In vitro* ploidy manipulation in the genomics era. PCTOC 104, 281–282.
Zuloaga, F.O., Morrone, O.N., Belgrano, M.J., Marticorena, C., Marchesi, E., 2008. Catálogo de las plantas vasculares del Cono. Sur. Monogr. Syst. Bot. Mo. Bot. Gard. 107 (1–3), i–xcvi, 3348 pp.