Tilianin content and morphological characterization of colchicine-induced autotetraploids in *Agastache mexicana* (#95988)

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Tilianin content and morphological characterization of colchicine-induced autotetraploids in *Agastache mexicana*

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Background. *Agastache mexicana* Linton & Epling subsp. *mexicana* (Lamiaceae) is an aromatic medicinal plant, characterized by high concentration of tilianin, a flavonoid with therapeutic potential in cardiovascular diseases. In this study, we have explored the use of colchicine for to obtain autotetraploid lines of *A. mexicana* and analyze their morphological characteristics. In addition, we aimed to identify polyploid plants with high content of tilianin. **Methods.** *In vitro* seedlings at the stage of cotyledon emergence were dipped in colchicine solution at 0.0 %, 0.1 %, 0.3 %, and 0.5 % (w/v) for 6, 12 and 24 hours. Seedlings were cultured on half-strength basal Murashige and Skoog medium supplemented with 20 g/L sucrose. After two months, the newly regenerated shoots from surviving seedlings were excised and grown individually in the same medium for multiplication and rooting. The ploidy level of all materials was verified through flow cytometry and chromosome counting before acclimatization and transfer to the greenhouse. The investigated characteristics included length, density and stomatal index, leaf area, chlorophyll content, flower size and color, and tilianin content measured by high performance liquid chromatography. **Results.** The most efficient production of tetraploid in terms of percentage was achieved with 0.1 % colchicine for 6 hours resulting in no generation of mixoploids. Tetraploid plants had twice the number of chromosomes (2n = 4x = 36) and nearly twice the total DNA content (2.660 ± 0.236 pg) of diploids. Most of the tetraploid *A. mexicana* plants showed variations in flower and leaf characteristics, when compared to the diploid controls. High-performance liquid chromatography analysis showed that tetraploid plants with small leaves produced the greatest amount of tilianin;

up to 32.964 ± 0.004 mg/g dry weight (DW), compared to diploid plants with 6.388 ± 0.005 mg/g DW. **Conclusion.** *In vitro* polyploidization using colchicine has the potential to improvement of medicinal constituents of *A. mexicana*. Its application has been shown to be effective in the production of elite tetraploid lines with increased tilianin production.

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Abstract

Background. *Agastache mexicana* Linton & Epling subsp. *mexicana* (Lamiaceae) is an aromatic medicinal plant, characterized by a high concentration of tilianin, a flavonoid with therapeutic potential in cardiovascular diseases. In this study, we have explored the use of colchicine to obtain autotetraploid lines of *A. mexicana* and analyze their morphological characteristics. In addition, we aimed to identify polyploid plants with a high content of tilianin.

Methods. *In vitro* seedlings at the stage of cotyledon emergence were dipped in colchicine solution at 0.0 %, 0.1 %, 0.3 %, and 0.5 % (w/v) for 6, 12, and 24 hours. Seedlings were cultured on half-strength basal Murashige and Skoog medium supplemented with 20 g/L sucrose. After two months, the newly regenerated shoots from surviving seedlings were excised and grown individually in the same medium for multiplication and rooting. The ploidy level of all materials was verified through flow cytometry and chromosome counting before acclimatization and transfer to the greenhouse. The investigated characteristics included length, density and stomatal index, leaf area, chlorophyll content, flower size and color, and tilianin content measured by high-performance liquid chromatography.

Results. The most efficient production of tetraploid in terms of percentage was achieved with 0.1 % colchicine for 6 hours resulting in no generation of mixoploids. Tetraploid plants had twice the number of chromosomes ($2n = 4x = 36$) and nearly twice the total DNA content (2.660 ± 0.236 pg) of diploids. Most of the tetraploid *A. mexicana* plants showed variations in flower and leaf characteristics when compared to the diploid controls. High-performance liquid chromatography analysis showed that tetraploid plants with small leaves produced the greatest amount of tilianin; up to 32.964 ± 0.004 mg/g dry weight (DW), compared to diploid plants with 6.388 ± 0.005 mg/g DW.

Conclusion. *In vitro* polyploidization using colchicine has the potential to improve of medicinal constituents of *A. mexicana*. Its application is effective in the production of elite tetraploid lines with increased tilianin production.

Introduction

Agastache mexicana Linton & Epling subsp. *mexicana* (Lamiaceae) is an aromatic plant, native to North America and widely cultivated in central Mexico for its medicinal and ornamental

properties (Palma-Tenango et al., 2021). The aerial parts of *A. mexicana* have been used in Mexican traditional medicine to treat a range of ailments, including insomnia, anxiety, rheumatism, stomach pain, gastrointestinal disorders, and cardiovascular disease (González-Ramírez et al., 2012; Flores-Flores et al. 2016). The medicinal properties of *A. mexicana* are predominantly attributed to the presence of terpenes and phenolic compounds, particularly tilianin (acacetin-7-glucoside), which is the most abundant flavonoid (9.77 mg/g dry weight) in this plant (Carmona-Castro et al., 2019). Tilianin is a bioactive compound derived from plant secondary metabolism that manifests various biological activities beneficial to human health, including neuroprotective, anti-atherogenic, anti-hypertensive, cardioprotective, anti-inflammatory, antioxidant, and anti-depressant effects, among others (Akanda et al., 2019). According to the pharmacological findings, tilianin could lead to the development of new drugs for the treatment of cardiovascular diseases (Khattulanuar et al., 2022; Cruz-Torres et al., 2023; Du et al., 2023). However, similar to other medicinal and aromatic plants, few efforts have been applied to increase the valuable secondary metabolites and ornamental characteristics of *A. mexicana*. One of the remarkable breeding strategies to improve plant desirable traits is artificial polyploidization or chromosome doubling by application of mitotic spindle inhibitors on somatic cells that results in morphological changes and has often been associated with increased production of bioactive secondary metabolites (Niazian, 2019; Niazian & Nalousi 2020). Colchicine is a plant alkaloid, known for its antimitotic activity and widely used in plant polyploidization. This compound has been successfully used for tetraploidy induction in the case of several genera from the Lamiaceae family, registering a significant effect on the content and composition of bioactive compounds in species of *Agastache* (Tabeli et al., 2017), *Dracocephalu*m (Yavari et al., 2011), *Lavandula* (Urwin et al., 2007; Urwin, 2014), *Ocimum* (Omidbaigi et al., 2010), *Salvia* (Estaji et al., 2017), and *Thymus* (Tavan et al., 2015). In the case of the *Agastache* genus, few studies have been published, which evaluate the effect of polyploidization on the qualitative and quantitative production of bioactive compounds. For instance, in *A. foeniculum*, the variation in ploidy level, significantly affected the physicochemical and morphological characteristics of the tetraploid plants. The polyploidy plants showed an increase in essential oil content and chemical composition, as well as an increased tolerance to salt stress (Talebi et al., 2016; 2017; 2021). Therefore, in the present study, we hypothesized that artificial chromosome doubling using colchicine could be an effective approach to obtaining new genotypes with high tilianin content. Hence, the aim was to obtain autotetraploid *A. mexicana* plants and to determine whether the manipulation of the ploidy level could be used to produce plant variants with higher tilianin content. This study also aimed to investigate the effect of polyploidization events on chlorophyll content, and some leaf and flower morphological characteristics in ten autotetraploid lines of *A. mexicana*.

Materials & Methods

Polyploid induction

A. mexicana seeds were surface sterilized using 98 % ethanol for 5 min, rinsed with sterile water and then with 1.5 % of commercial sodium hypochlorite (Cloralex®) for 2 min. Subsequently, the seeds were rinsed five times in sterile water and germinated on half-strength basal Murashige and Skoog medium (MS 50 %), containing 50 mg/L myo-inositol and 3.0 g/L Phytagel®. All media were adjusted to pH 5.8 ± 0.1 and autoclaved at 121 °C for 25 min. The cultures were maintained at 25 ± 1 °C under a light regime of 16/8 h (light/dark) photoperiod with a light intensity of 2000 lux.

After eleven days of culture, the seedlings at the stage of cotyledon emergence were dipped in 0.0, 0.1, 0.3, and 0.5 % (w/v) colchicine solution for 6, 12 and, 24 h. Colchicine was sterilized by filtration through a 0.22 µm Millipore syringe filter. Each treatment consisted of 12 seedlings that were incubated with constant shaking at 100 rpm, in the dark at 25 °C to avoid deterioration of the antimitotic agent under light (Eng et al., 2021). Dimethyl sulfoxide (DMSO) was added at a non-toxic concentration of 2 % to help the colchicine penetrate through cell walls (Głowacka et al., 2009; Salma et al., 2017). At the end of each treatment, the seedlings were rinsed eight times with sterile water and then transferred to the same medium, supplemented with 20 g/L sucrose. After two months, the newly regenerated shoots (10 cm length) from surviving seedlings were individually excised and cultured for multiplication and rooting into 250 mL glass jars, containing 50 mL of MS 50 % medium and subcultures, every 20 days. The jars were sealed with aluminum foil caps with tiny holes covered with a piece of 3M Micropore™ tape that enables reducing humidity in the jars, while increasing gas exchange to minimize the effect caused by hyperhydricity or vitrification (Zarate-Salazar et al., 2020). The cultures were incubated under the same conditions as described previously. Plantlets obtained by *in vitro* culture of single shoots from colchicine-treated and untreated seedlings were identified as putative polyploid lines (A*mx*) and diploid control, respectively. After 8 weeks of culture, the length, density, and stomatal index of micropropagated plantlets were assessed. Chromosome duplication was confirmed by chromosome counts in young root tips and flow cytometry of leaf nuclei. After 15 months of successive subcultures and confirmed the ploidy level, diploid controls and tetraploid lines were transferred to soil and grown under greenhouse conditions, as described by Carmona-Castro et al. (2019). At least five plants per line were grown and subsequently leaf area, chlorophyll content, flower characteristics and tilianin content were analyzed.

Stomatal characteristics

Five fully expanded leaves were excised at node position 3 from the shoots of diploid control and from five randomly selected putative polyploid lines of the *in vitro* cultures. The abaxial leaf surfaces were coated with a thin layer of nail polish. After 10 min, the dried polish was removed by applying a strip of transparent one-sided adhesive tape. The dry polish samples, along with the adhered sticky tape were mounted permanently on glass microscope slides, and the stomata length, stomata density (number of stomata per visual field, PVF), and stomata index were

recorded using a Leica DM500 optical microscope (Leica Microsystems, Germany). Images were analyzed using image J software (https://imagej.nih.gov/ij/). The fields of view were located in the middle portion of leaf lamina, and three fields of vision were investigated for each leaf. The stomatal index was determined from the formula: SI = [S/(S+E)] x 100, where S is the number of stomata in the microscopic field and E is the number of epidermal cells per unit leaf area (Mishra 1997). Data is presented as the mean of 15 observations for length, density and stomatal index.

Chromosome count

Mitotic chromosomes were prepared from young root meristems, following the method described by Las Peñas et al. (2008), with minor modifications. *In vitro* root tips (~0.5-1 cm) were excised from putative polyploid lines and diploid controls between 7 and 8 o'clock in the morning and pretreated with 0.002 mM 8-hydroxyquinoline (8-HQ) for 24 h at 4 °C in the dark and rinsed with distilled water for 5 min to remove 8-HQ. The root tips were then fixed in Farmer's solution (ethyl alcohol: acetic acid, 3:1 v/v) for 24 h at room temperature and rinsed with distilled water. Subsequently, samples were hydrolyzed in 1N HCl at 60 °C for 10 min and stained with Schiff's reagent for 1 h in the dark. Finally, each hydrolyzed root was crushed in a drop of 45 % (w/v) acetocarmine and 45 % (w/v) acetic acid, and the number of chromosomes in mitotic cells was determined using a light microscope at 100x magnification (DM500®, Leica Microsystems, Germany). A total of 10 representative photomicrographs were analyzed from three root tips from each line, including the control.

Flow cytometry

Fresh apical leaves from the selected *in vitro* lines, including the control, were chopped in 1.5 mL Galbraith's modified buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 4-morpholine propane sulfonate (MOPS) and 0.5 % (v/v) TritonX-100, pH 7.0) (Galbraith et al., 1983). After filtration through a 30 µm nylon mesh, crude nuclear samples were stained with 10 mg of propidium iodide. Nuclear DNA content was determined, using the method described by Arumuganathan and Earle (1991), which employs an Attune® Acoustic Focusing Flow Cytometer blue/violet (Applied Biosystems, United States of America). Fresh leaves from *Solanum lycopersicum* (2C = 1.96 pg DNA) were used as an internal standard (Doležel et al., 2007) and more than 5000 nuclei per sample were analyzed. Three independent replicates were performed for each analysis.

Floral characteristics

To study the effect of polyploidy on some floral characteristics, the size of the flower, as well as the color were assessed in ten tetraploid lines and a control. One inflorescence at peak bloom was randomly selected from each line for measurements. Eighteen individual, fully opened flowers from the top, middle, and bottom of the inflorescence were selected for flower length and maximum calyx length, using a digital vernier caliper. Color coordinates were performed

using a sprectrophotometer (X-Rite SP64, USA) (McGuire, 1992). The X-Rite SP64 was positioned with minimal pressure, perpendicular to the lower lip of each flower and the data were reported in the *L** (luminosity 0 = black, 100 = white), *C** (chromaticity, saturation level of *h*) and *h* (tone angle: 0° = red, 90° = yellow, 180° = green, 270° = blue, 300° magenta) colorimetric system according to Commission Internationale De L'eleirage (CIE) (Commission Internationale De L'ecleirage, 2004). For accuracy comparison Royal Horticultural Society (RHS) Colour Charts were used to compare with the X-Rite SP64 sensor readings. Data reported represent the averages for three measurements per flower.

Leaf area and chlorophyll content

Ten tetraploid lines were evaluated under greenhouse conditions to assess the effect of polyploidy on leaf area and chlorophyll content. Leaf area was determined with a leaf area meter (LI-3100C AREA METER, LI-COR® Bio Sciences Instrument, USA) and chlorophyll content (Chl *a*, *b* and total) using a portable chlorophyllmeter ClorofiLOG (model CFL 1030, Falker, Brazil). For each line and control (diploid), five plants were selected at random, and triplicate measurements were performed on two fully developed leaves, taken from the middle section of the shoots.

Quantification of Tilianin by HPLC

The quantification of tilianin from tetraploid and diploid lines was based on the method established by Hernández-Abreu et al. (2009). For each line, 10 g of finely ground dried aerial part plant material was subjected to continuous maceration (1:10 w/v) with hexane (C_6H_{14}), dichloromethane (CH_2Cl_2), and methanol (CH_30H) three times for 72 h at room temperature. The tilianin content was determined in the methanolic extract using Waters HPLC equipment with a photodiode array detector, Zorbax C18 SB-CN (4.6 mm × 250 mm, 5-µm particle size, Agilent®), and data analysis was performed using Empower 2002 software. The mobile phase consisted of methanol-water at a ratio of 61:39 (v/v) at a flow rate of 0.7 ml/min and a wavelength of 260 nm. The quantification of tilianin was defined according to the corresponding calibration curve at concentrations of 103.258, 34.264, 27.693, 20.173, 13.027, and 6.442 µg/mL, using highly purified tilianin as reference. All samples were assayed in triplicate.

Statistical analysis

The effect of different concentration of colchicine (0.0, 0.1, 0.3, 0.5 %) and exposure time (6, 12, 24 h) on seedling survival rate was analyzed by applying a multiple linear regression (Rossi, 2022). To determine possible phenotypic differences between ten polyploid lines (genotypes) and diploid control, we performed One-way Analysis of Variance (ANOVA) to define stomata length, stomata density, stomata index, leaf area, chlorophyll a, chlorophyll b, total chlorophyll and tilianin content. Differences were analyzed by applying the Tukey multiple comparisons test ($P < 0.05$). Principal Component Analysis (PCA) was carried out to determine potential relationships between genome size and phenotypic traits, previously mentioned. All analyses

were performed in R version 4.2.3 (R Core Team, 2023) using ade4 (PCA, Dray, S. & Dufour 2007) and ggplot2 (Wickham, 2016) libraries, which were used to generate graphs.

Results

Identification of tetraploids and ploidy stability

The first visible effect of colchicine was reflected in the browning and delayed growth rate of treated seedlings. Significant differences between treatments were observed after 2 weeks of culture, and multiple linear regression analysis [y = 1.278-colchicine (2.279)-time exposure (0.005)] revealed a strong negative correlation between seedling survival and colchicine dosage ($p = 0.003$), but no significant correlation was found with exposure time. The second effect of colchicine observed was the inhibition of shoot multiplication and *in vitro* rooting. The number of shoots per seedling decreased with increasing colchicine dosage, so the mean value of regenerated shoots on colchicine-treated and untreated (diploid control) by explant was 0.40 ± 0.329 and 1.79 ± 0.318, respectively. Overall, the higher the colchicine dose, the lower the survival rate and number of regenerated plantlets (lines) (Table 1).

In this study, 29 putative polyploid lines of *A. mexicana* were obtained from colchicine treatments. Generally, after 8 weeks of *in vitro* culture, the newly regenerated plantlets showed markedly different morphological characteristics from the diploid controls, manifesting smaller or larger leaves, often with a rolled structure, and ranging in color from purple to dark green color. Significant differences were observed between the stomatal characteristics of the *in vitro* diploid control and tetraploid lines. As shown in Figure 1, the leaves from tetraploid lines exhibited large stomata, with significantly higher stomatal lengths than leaves from diploid control ($F_{5,24} = 4.613$, $p < 0.01$; 4x = 37.94 ± 7.81 μm; 2x = 31.46 ± 4.00 μm), as well as, a lower stomatal density ($F_{5,24} = 21.82$, $p < 0.001$; 4x = 117.5 ± 60.26 PVF; 2x = 161.24 ± 19.43 PVF) and stomatal index ($F_{5,24} = 18.47$, $p < 0.001$; 4x = 10.25 ± 2.22 %; 2x = 16.23 ± 1.85 %).

Studies of mitotic cells in actively growing root tips clearly indicated that the polyploidy of the samples was due to chromosome doubling in diploid seedlings induced by colchicine treatments. Figure 2 shows that the number of chromosomes in the polyploid plantlets is 2n = 4x = 36, whereas the number of chromosomes in the diploid plantlets (controls) is 2n = 2x = 18. Of the 29 lines evaluated; 23 (79.3 %) were tetraploids, 5 (17.3 %) diploids, and 1 (3.4 %) mixoploid, containing a mixture of diploid and tetraploid cells. In order to confirm the ploidy level of regenerated plantlets using flow cytometry, as there have been no reports to date that estimate genome size of *A. mexicana*, it was necessary to determine the genomic content, by comparing the mean position of the G0/G1 peak of the internal standard of *S. lycopersicum* with the mean position of the peak of the diploid sample of *A. mexicana*. Eight independent measurements were performed to conclude that diploid control plants (2n = 18) have an average genomic content of 1.433 ± 0.025 pg. Flow cytometry DNA histograms of the polyploid lines revealed that the peak position was twice that of the genome DNA of diploid (Figure 3). It was thus concluded that the

DNA content of the tetraploid cells (2.660 ± 0.236 pg) equals twice that of diploid cells. The three groups of ploidy levels: diploid, tetraploid, and mixoploid plantlets based on flow cytometry, fully concurred with the results obtained from counting chromosomes. The highest percentage of tetraploid production efficiency (91.66 %) was achieved with 0.1 % colchicine for 6 hours, without generating any mixoploids (Table 1). Chromosome counting and flow cytometry analysis indicated that DNA ploidy levels remain stable in all tetraploid lines after 15 months of *in vitro* culture.

During the *in vitro* culture, the mixoploid line and several tetraploid lines showed symptoms of hyperhydricity, low growth capacity, and reduced root development. However, after acclimatization to greenhouse conditions, ten tetraploid lines exhibited accelerated growth and development, compared to the diploid control. These lines were selected to evaluate the effect of ploidy on leaf and flower traits, and on tilianin content in *A. mexicana*.

Flower and leaf characteristics of *A. mexicana* grown under greenhouse condition

Inflorescence emergence began earlier in the tetraploid plants; after only 3 months in the greenhouse, compared to 4 months for diploid control. All tetraploid lines, except for line A*mx*2, exhibited significantly increased flower and calyx lengths compared to the diploid control (Table 2). In tetraploid lines, the mean lengths of flowers and calyxes were 30.659 ± 2.401 mm and 11.732 ± 0.801 mm, respectively, so significantly greater than those in diploid lines (25.252 ± 2.947 mm and 11.271 ± 2.334 mm, respectively; $p < 0.0001$). Also, there were significant differences in the color components of flowers of tetraploids and diploid control (Table 2). However, in both tetraploid lines and diploids, the value of *L** tends to be neutral, the color tends to be magenta and slightly more opaque (*C**= less than 30) in the tetraploid lines, compared to the diploid control, which tends to be violet. This resembles the visual evaluation provided by the RHS, which identified the color of the diploid flowers as deep strong reddish purple C (NN78) and the tetraploid flowers as light reddish purple D (NN78).

Differences in leaf area were observed between the diploid and the tetraploid lines A*mx*3, A*mx*7, and A*mx*10, with the tetraploids manifesting a higher chlorophyll content and smaller leaf area (Figure 1, Table 3). Tetraploid plants presented a dark green leaf color.

Tilianin content

Statistically significant differences in tilianin content were found between the tetraploid lines and diploid control in methanolic extract ($F_{10, 22} = 23806$, $p < 0.0001$). The diploid control produced an average of 6.388 ± 0.005 mg of tilianin per gram of dry weight (mg/g DW). Out of the tetraploid lines, the greatest tilianin accumulation was recorded in A*mx*3 (32.964 ± 0.004 mg/g DW) and A*mx*7 (32.392 ± 0.110 mg/g DW). Compared to the control (as shown in Figure 4), A*mx*8 (3.195 ± 0.005 mg/g DW) exhibited less accumulation of tilianin.

Principal component analysis

Principal component analysis (PCA) was performed to investigate how the patterns of variance of morphological traits and tilianin content correlated with ploidy level. This was also used to determine their possible contribution to the content of tilianin and to identify particular traits that distinguish diploid from tetraploid plants. Two principal components, PC1 and PC2, were determined, based on their degree of contribution. The first two components accounted for 57.05 % of total variance, with 33.88 % relating to PC1 and 23.17 % relating to PC2 (Figure 5). PC1 correlated positively with C^* (0.711) and negatively with calyx length (-0.805), DNA (-0.796), flower length (-0.760) and h (-0.498). PC2 showed a positive correlation with tilianin (0.714), and negative correlation with L (-0.748) and leaf area (-0.589). The control group is separated from the tetraploid lines in Figure 5b.

Discussion

Antimitotic agents such as colchicine have been used in medicinal plant breeding for artificial *in vitro* polyploidy induction and the development of varieties with improved agronomic traits, enhanced abiotic stress tolerance, and high levels of bioactive secondary metabolites. However, the phenotypic and genetic changes in plants that occur due to the artificial chromosome doubling are often unpredictable and can differ significantly between species (Niazian & Nalousi 2020; Tavan et al., 2022). In this study, the feasibility and efficacy of colchicine for polyploidization of *A. mexicana* and the generation of polyploid lines with high tilianin production were investigated. For this purpose, we follow the techniques previously applied to other plant species, such as *Platanus aceriolia* (Liu et al., 2007), *Arabidopsis thaliana* (Yu et al., 2009), and *Agastache foeniculum* (Talebi et al., 2017). *In vitro*-cultured young seedlings of *A. mexicana* were directly treated with one of four colchicine concentrations (0.0, 0.1, 0.3, and 0.5 %, w/v) for 6, 12 and, 24 h. The results from the present study revealed that the survival of seedlings and the efficiency of tetraploid lines recovery were affected by the concentration of this antimitotic agent but not by the duration of exposure (Table 1). Some studies suggest that colchicine has a low affinity for plant tubulin, requiring relatively high concentrations to disrupt microtubule formation and consequently promote polyploidization (Hailu et al., 2021). However, high concentrations of colchicine have been shown to alter gene expression, including genes related to the phenylpropanoid biosynthetic pathways and plant hormone signaling. This alteration may contribute to explant mortality during chromosome doubling and low plantlet regeneration ability caused by the death of meristematic cells (Temel & Gozukirmizi, 2015; Zhou et al., 2017). It is therefore paramount to carefully select a suitable colchicine concentration to achieve a high polyploid induction rate. Indeed, some research suggests that factors, such as plant genotype, ecotypes, type and age of explants are also important for improving artificial polyploidy induction efficiency (Salma et al., 2017; Niazian & Nalousi, 2020). In this study, the most efficient treatment for tetraploid induction of *A. mexicana* was observed to be 0.1 % colchicine for 6 hours, with a 91.66 % explant survival rate and 58.33 % tetraploidy induction. These results concur with previous studies showing that colchicine

concentrations used for the induction of polyploid plants usually range from 0.005 % to 1.0 % (w/v), and the duration of treatment takes from hours to weeks, depending on application methods (Eng & Ho, 2019; Ahmandi & Ebrahimzadeh, 2020).
Microscopy examination of meristematic cells showed that the diploid plants of *A. mexicana* have a chromosome number of 2n = 18, whereas the tetraploid lines plants have 4n = 36. This result concurred with the previous report of a haploid number n = 9 for *A. mexicana* (Sander 1987). Polyploidy was confirmed using flow cytometry, a method which proved to be fast and accurate for estimating the increase in DNA content in *A. mexicana*. The DNA content estimated for diploid plants (2C = 1.433 ± 0.025 pg) and tetraploid plants (2.660 ± 0.236 pg) of *A. mexicana* was similar to that reported for *A. foeniculum*. As far as we know, among the *Agastache genus*, *A. foeniculum* is the only species for which the genome sizes have been reported; with means of 1.06 ± 0.02 pg for diploid 2C value and 2.15 ± 0.001 pg for tetraploid plants (Talebi et al., 2016).

Studies on several species suggest that the extra set of chromosomes in polyploid plants will often, though not always, lead to increased biomass or content of bioactive secondary metabolites, by means of changes in gene transcription, epigenetic modifications, and morphological and physiological alterations (Osbor et al., 2003; Lavania 2013; Iannicell et al., 2020); therefore, the phenotypic variations generated by the polyploidization may help distinguish diploid from polyploid plants. In this study, under *in vitro* conditions, the results indicated that an initial screening on the basis of stomata size might be effective for identifying putative polyploids. According to Beaulieu et al. (2008) there is a positive correlation between genome size and guard cell length and a negative correlation between stomatal size and stomatal density. This concurs with the results presented here, indicating that an increase in ploidy level increases stomatal length and decreases stomatal density and stomatal index in *A. mexicana* autotetraploid plants. Similar to previous studies reported in tetraploid plants of *Hibiscus syriacus* (Lattier et al., 2019) and *A. foeniculum* (Talebi et al., 2017).
The diploid control, tetraploid, and mixoploid lines showed slight symptoms of hyperhydricity, a common problem for *in vitro* cultures (Gao et al., 2017), which may have affected rooting and greenhouse acclimatization. Hyperhydricity also resulted in delayed development of the only mixoploid line obtained, which then ceased to grow after a brief period of time. Consequently, only the stable tetraploid plants, confirmed by flow cytometry after 15 months of successive *in vitro* subcultures were selected for transfer to the greenhouse to determine leaf area, chlorophyll content, flower characteristics, and tilianin content.
The PCA analysis indicates a positive correlation between flower size and DNA content of tetraploid plants under greenhouse conditions. *A. mexicana* tetraploid plants exhibited larger flowers than their diploid counterparts. Flowers from diploids plants were smaller and tended to be purple compared to tetraploid plants, which tended to be magenta. Previous findings reported flower color modifications among tetraploid plants of *Rosa centifolia*, *Impatiens walleriana* and, *Gladiolus grandiflorus* probably, resulting from alterations in the biosynthesis pathway of

pigments (e.g. anthocyanin) and increased size of cells due to chromosome doubling effect (Osborn et al., 2003; Manzoor et al., 2018; Ghanbari et al., 2019). It is generally accepted that the cells in polyploid plants tend to be larger than those in their corresponding diploid plants, resulting in thicker and bigger leaves, as well as larger flowers and fruits (Hu et al. 2021). Increase in flower size has been observed in the autopolyploid plants of several species, including *Crocosmia aurea* (Hannweg 2013), *Gerbera jamesonii* Bolus cv. Sciella (Gantait et al., 2011), *Hemerocallis* x *hybrida* 'Blink of an Eye´ (Podwyszynska et al., 2015) and, *Vicia villosa* (Tulay & Unal, 2010). However, in most tetraploid lines of *A. mexicana* with large flowers, there was a tendency for the leaf area to decrease, as shown by PCA analysis (Figure 5). Previous studies have noted that among certain species, including *Rhododendron furtunei* (Mo et al., 2020), *Escallonia rosea* (Denaeghel et al., 2018), *Gladiolus grandiflorus* (Manzoor et al., 2018), and *Arabidopsis thaliana*, the leaf area of tetraploid and octoploid variants decreases as their ploidy level increases. According to Iannicelli et al. (2020), the polyploid cells may expand to maintain the balance between cytoplasmic and nuclear volume, which is necessary for efficient cellular machinery function, due to an increased DNA content in the nucleus. This results in a delay in the growth of tissues and organs due to higher energy expenditure and a decrease in the surface area to volume ratio of polyploid cells, thereby reducing growth and cell division (Tsukaya 2008; Agafarini et al., 2019).

On the other hand, a large number of studies have demonstrated that changes in plant ploidy affect the gene transcripts, protein synthesis, and photosynthetic elements, which often result in beneficial impact to secondary metabolite biosynthesis pathways (Lavania et al., 2012; Gantait & Mukherjee, 2021). Interestingly, PCA analysis revealed that DNA content did not correlate with the total chlorophyll and tilianin content in *A. mexicana*. However, this statistical analysis demonstrated a significant negative correlation between the accumulation of tilianin and leaf area (Figure 5). The tetraploid lines A*mx*3, A*mx*7 and A*mx*10, which have the smallest leaf area, showed higher tilianin content than diploid plants. In contrast, plants with larger leaves had lower tilianin accumulation than the diploid controls (Table 3). Therefore, the measurement of leaf area is an essential selection criterion for high content tilianin content in tetraploid plants of *A. mexicana*. Powell et al. (2015) reported that biomass changes in biosynthetic tissues of polyploid plants may either increase or decrease flavonoid production. Both qualitative and quantitative variations in flavonoids levels have been detected in various autotetraploid plants, including, *Chamomilla recutita*, *Isatis indigotica*, and *Polemonium caeruleum* (Repák 2000; Zhang et al., 2021; Samatadze et al., 2022). However, the molecular mechanisms that explain the association between polyploidy and flavonoid metabolism remain undefined. Polyploidy involves more than just chromosome doubling and always results in changes to genome structure and gene expression. Information about the morphological traits of polyploid plants of *Agastache* genus and their effect on phenolic and flavonoid compounds remains limited. In the future, the study of genetic variation in the expression of gene encoding enzymes involved in the tilianin pathways will shed light on the regulatory mechanism by which the ploidy level affects the flavonoids content in *A. mexicana*.

Conclusions

This study demonstrated that ploidy manipulation via *in vitro* chromosome doubling represents a workable approach for the improvement of *A. mexicana*, and its application shows potential for the production of elite tetraploid lines with increased tilianin production.

Acknowledgements

The authors are thankful to Ariadna Zenil Rodríguez from Centro de Investigación en Biotecnología for her help with the HPLC Waters, as well as to Doctorado en Ciencias Naturales de la Universidad Autónoma del Estado de Morelos. The authors appreciate the collaboration of Laboratorio Nacional de Investigación y Servicio Agroalimentario y Forestal, Universidad Autónoma de Chapingo and the technical support of Juan Carlos Cervantes Gutiérrez of Flow Cytometry Area.

References

Ahmadi B, Ebrahimzadeh H. 2020. *In vitro* androgenesis: spontaneous vs. artificial genome doubling and characterization of regenerants. *Plant Cell Reports*, 39(3): 299-316 DOI: 10.1007/s00299-020-02509-z.

Akanda MR, Uddin MN, Kim IS, Ahn D, Tae HJ, Park BY. 2019. The biological and pharmacological roles of polyphenol flavonoid tilianin. *European Journal of Pharmacology*, 842: 291-297. DOI: 10.1016/j.ejphar.2018.10.044.

Aqafarini A, Lotfi M, Norouzi M, Karimzadeh, G. 2019. Induction of tetraploidy in garden cress: morphological and cytological changes. *Plant Cell, Tissue and Organ Culture*, 137: 627-635. DOI: 10.1007/s11240-019-01596-5.

Arumuganathan K, Earle ED. 1991. Estimation of nuclear DNA content of plants by flow cytometry *Plant Molecular Biology Reporter*, 9: 229-241. DOI: 10.1007/BF02672073.

Beaulieu JM, Leitch IJ, Patel S, Pendharkar A, Knight CA. 2008. Genome size is a strong predictor of cell size and stomatal density in angiosperms. *New Phytologist*, 179(4): 975-986. DOI: 10.1111/j.1469-8137.2008.02528.x.

Carmona-Castro G, Estrada-Soto S, Arellano-García J, Arias-Duran L, Valencia-Díaz S, Perea-Arango I. 2019. High accumulation of tilianin in *in-vitro* cultures of *Agastache mexicana* and its potential vasorelaxant action. *Molecular Biology Reports*, 46:1107-1115. DOI: 10.1007/s11033-018-4570-4

Cruz-Torres KC, Estrada-Soto S, Arias-Durán L, Navarrete-Vázquez G, Almanza-Pérez JC, Mora-Ramiro B, Perea-Arango I, Hernández-Núñez E, Villalobos-Molina R, Carmona-Castro G, Medina-Díaz IM, Ávila-Villarreal G. 2023. LC-MS Fingerprinting Development for Standardized Precipitate from *Agastache mexicana*, Which Induces Antihypertensive Effect through NO Production and Calcium Channel Blockade. *Pharmaceutics*, 15(9): 2346. DOI: 10.3390/pharmaceutics15092346.

Denaeghel HE, Van Laere K, Leus L, Lootens P, Van Huylenbroeck J, Van Labeke MC. 2018. The variable effect of polyploidization on the phenotype in *Escallonia*. *Frontiers in Plant Science*, *9*: 354. DOI: 10.3389/fpls.2018.00354.

Doležel J, Greilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols*, 2(9): 2233-2244. DOI: 10.1038/nprot.2007.310.

Dray S, Dufour A. 2007. The ade4 Package: Implementing the Duality Diagram for Ecologists. J. Stat. *Software 22*: 1-20. DOI: 10.18637/jss.v022.i04.

Du Y, Xi M, Li Y, Zheng R, Ding X, Li X, Zhang X, Wang L, Xing J, Hong B. 2023. Tilianin improves lipid profile and alleviates atherosclerosis in ApoE−/− mice through up-regulation of SREBP2-mediated LDLR expression. *Phytomedicine*, 109: 154577. DOI: 10.1016/j.phymed.2022.154577.

Eng WH, Ho WS. 2019. Polyploidization using colchicine in horticultural plants: A review. *Scientia Horticulturae*, 246: 604-617. DOI:10.1016/j.scienta.2018.11.010.

Eng WH, Ho WS, Ling KH. 2021. *In vitro* induction and identification of polyploid *Neolamarckia cadamba* plants by colchicine treatment. *PeerJ*, 9:e12399. DOI: 10.7717/peerj.12399.

Estaji A, Hosseini B, Ghotbi Ravandi E, Dehghan E, Sefidkon F. 2017. The effects of colchicine-induced autotetraploidy on selected characteristics of nuruozak (*Salvia leriifolia*). *Cytology and Genetics*, 51: 74–81. DOI 10.3103/S0095452717010042.

Flores-Flores A, Hernández-Abreu O, Rios MY, León-Rivera I, Aguilar-Guadarrama B, Castillo-España P, Perea-Arango, I, Estrada-Soto S. 2016. Vasorelaxant mode of action of dichloromethane-soluble extract from *Agastache mexicana* and its main bioactive compounds. *Pharmaceutical Biology*, 54 (12) : 2807-2813. DOI: 10.1080/13880209.2016.1184690.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science*, 220(4601): 1049-1051. DOI: 10.1126/science.220.4601.1049.

Gantait S, Mandal N, Bhattacharyya S, Das PK. 2011. Induction and identification of tetraploids using *in vitro* colchicine treatment of *Gerbera jamesonii Bolus* cv. Sciella. *Plant Cell, Tissue and Organ Culture*, 106: 485-493. DOI: 10.1007/s11240-011-9947-1.

Gantait S, Mukherjee E. 2021. Induced autopolyploidy—a promising approach for enhanced biosynthesis of plant secondary metabolites: an insight. *Journal of Genetic Engineering and Biotechnology*, 19:1-13. DOI: 10.1186/s43141-020-00109-8.

Gao H, Xia X, An L, Xin X, Liang Y. 2017. Reversion of hyperhydricity in pink (*Dianthus chinensis L.)* plantlets by $AgNO_3$ and its associated mechanism during *in vitro* culture. *Plant Science*, 254:1-11. DOI: 10.1016/j.plantsci.2016.10.008.

Ghanbari MA, Jowkar A, Salehi H, Zarei M. 2019. Effects of polyploidization on petal characteristics and optical properties of *Impatiens walleriana* (Hook.). *Plant Cell, Tissue and Organ Culture*, 138: 299-310. DOI: 10.1007/s11240-019-01625-3.

Głowacka K, Jeżowski S, Kaczmarek Z. 2009. Polyploidization of *Miscanthus sinensis* and *Miscanthus* x *giganteus* by plant colchicine treatment. *Industrial Crops and Products*, 30(3): 444-446. DOI: 10.1016/j.indcrop.2009.07.011.

González-Ramírez A, González-Trujano ME, Pellicer FJ, López-Muñoz F. 2012. Anti-nociceptive and anti-inflammatory activities of the *Agastache mexicana* extracts by using several experimental models in rodents. *Journal of Ethnopharmacology*, 142(3): 700-705. DOI: 10.1016/j.jep.2012.05.044.

Hailu MG, Mawcha KT, Nshimiyimana S, Suharsono S. 2021. Garlic micro-propagation and polyploidy induction *in vitro* by colchicine. *Plant Breeding and Biotechnology*, 9(1): 1-19. DOI: 10.9787/PBB.2021.9.1.1.

Hannweg K, Sippel A, Bertling I. 2013. A simple and effective method for the micropropagation and *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea*. *South African Journal of Botany*, 88: 367-372. DOI: 10.1016/j.sajb.2013.09.005.

Hernández-Abreu O, Castillo-España P, León-Rivera I, Ibarra-Barajas M, Villalobos-Molina R, González-Christen J, Vergara-Galicia J, Estrada-Soto S. 2009. Antihypertensive and

vasorelaxant effects of tilianin isolated from *Agastache mexicana* are mediated by NO/cGMP pathway and potassium channel opening. *Biochemical Pharmacology*, 78(1): 54-61. DOI: 10.1016/j.bcp.2009.03.016.

Hu Y, Sun D, Hu H, Zuo X, Xia T, Xie J. 2021. A comparative study on morphological and fruit quality traits of diploid and polyploid carambola (*Averrhoa carambola L.*) genotypes. *Scientia Horticulturae*, 277: 109843. DOI: 10.1016/j.scienta.2020.109843.

Iannicelli J, Guariniello J, Tossi VE, Regalado JJ, Di Ciaccio L, Van Baren CM, Pitta Alvarez, SI, Escandón AS. 2020. The "polyploid effect" in the breeding of aromatic and medicinal species. *Scientia Horticulturae*, 260:108854. DOI: 10.1016/j.scienta.2019.108854.

Kasmiyati S, Kristiani EBE, Herawati MM. 2020. Effect of induced polyploidy on plant growth, chlorophyll and flavonoid content of *Artemisia cina*. Biosaintifika: *Journal of Biology & Biology Education*, 12(1): 90-96. DOI: 10.15294/biosaintifika.v12i1.22548.

Khattulanuar FS, Sekar M, Fuloria S, Gan SH, Rani NNIM, Ravi S, Chidambaram K, Begum MY, Azad AK, Jeyabalan S, Dhiravidamani A, Thangavelu L, Lum PT, Subramaniyan V, Wu YS, Sathasivam KV, Fuloria NK. 2022. Tilianin: a potential natural lead molecule for new drug design and development for the treatment of cardiovascular disorders. *Molecules*, 27(3): 673. DOI: 10.3390/molecules27030673.

Las Peñas ML, Bernardello G, Kiesling R. 2008. Karyotypes and fluorescent chromosome banding in *Pyrrhocactus* (Cactaceae). *Plant Systematics and Evolution*, 272: 211-222. DOI: 10.1007/s00606-007-0611-5.

Lattier JD, Chen H, Contreras RN. 2019. Variation in genome size, ploidy, stomata, and rDNA signals in Althea. *Journal of the American Society for Horticultural Science*, 144(2): 130-140. DOI: 10.21273/JASHS04618-18.

Lavania UC. 2013. Polyploidy, body size, and opportunities for genetic enhancement and fixation of heterozygosity in plants. *The Nucleus*, 56(1):1-6. DOI: 10.1007/s13237-013-0075-7.

Lavania UC, Srivastava S, Lavania S, Basu S, Misra NK, Mukai Y. 2012. Autopolyploidy differentially influences body size in plants, but facilitates enhanced accumulation of secondary metabolites, causing increased cytosine methylation. *The Plant Journal*, 71(4): 539-549. DOI: 10.1111/j.1365-313X.2012.05006.x.

Liu G, Li Z, Bao M. 2007. Colchicine-induced chromosome doubling in *Platanus acerifolia* and its effect on plant morphology. *Euphytica*, 157: 145-154. DOI: 10.1007/s10681-007-9406-6.

Lubbe A, Verpoorte R. 2011. Cultivation of medicinal and aromatic plants for specialty industrial materials. *Industrial Crops and Products*, 34(1): 785-801. DOI: 10.1016/j.indcrop.2011.01.019.

Manzoor A, Ahmad T, Bashir MA, Baig MMQ, Quresh AA, Shah MKN, Hafiz IA. 2018. Induction and identification of colchicine induced polyploidy in *Gladiolus grandiflorus* 'White Prosperity'. Folia *Horticulturae*, 30(2): 307-319. DOI: 10.2478/fhort-2018-0026.

Mishra MK. 1997. Stomatal Characteristics at Different Ploidy Levels in *Coffea L*. Annals of Botany, 80(5): 689-692. DOI: 10.1006/anbo.1997.0491.

Mo L, Chen J, Lou X, Xu Q, Dong R, Tong Z, Huang H, Lin E. 2020. Colchicine-induced polyploidy in *Rhododendron fortunei* Lindl. *Plants*, 9(4): 424. DOI: 10.3390/plants9040424.

Niazian M. 2019. Application of genetics and biotechnology for improving medicinal plants. *Planta*, 249: 953-973. DOI: 10.1007/s00425-019-03099-1.

Niazian M, Nalousi AM. 2020. Artificial polyploidy induction for improvement of ornamental and medicinal plants. *Plant Cell, Tissue and Organ Culture*, 142(3): 447-469. DOI: 10.1007/s11240-020-01888-1.

Omidbaigi R, Mirzaei M, Moghadam MS. 2010. Difference of growth traits, essential oil content and compositions between diploid and iinduced tetraploid plants of basil (*Ocimum basilicum* L.). *Journal of Essential Oil Bearing Plants*, 13(5): 579-587. DOI: 10.1080/0972060X.2010.10643866.

Osborn TC, Pires JC, Birchler JA, Auger DL, Chen ZJ, Lee HS, Comai L, Madlung A, Doerge RW, Colot V, Martienssen RA. 2003. Understanding mechanisms of novel gene expression in polyploids. *Trends in Genetics*, 19(3): 141-147. DOI: 10.1016/S0168-9525(03)00015-5.

Palma-Tenango M, Sánchez-Fernández RE, Soto-Hernández M. 2021. A systematic approach to *Agastache mexicana* research: biology, agronomy, phytochemistry, and bioactivity. *Molecules*, 26(12): 3751. DOI: 10.3390/molecules26123751.

Podwyszyńska M, Gabryszewska E, Dyki B, Stępowska AA, Kowalski A, Jasiński A. 2015. Phenotypic and genome size changes (variation) in synthetic tetraploids of daylily (*Hemerocallis*) in relation to their diploid counterparts. *Euphytica*, 203:1-16. DOI: 10.1007/s10681-014-1212-3.

638 Powell AF, Doyle JJ. 2015. The implications of polyploidy for the evolution of signalling in
639 rhizobial nodulation symbiosis. In *Advances in Botanical Research,* 75: 149-190. DOI:
640 10.1016/bs.abr.2015.09.007.
641 Repák M. 2000. Variation of apigenin quantity in diploid and tetraploid *Chamomilla recutita* (L.)
642 Rauschert. *Plant Biology*, 2(04): 403-407. DOI: 10.1055/s-2000-5957.
643
644 RHS Colour Chart. 2015. Royal Horticultural Society (RHS) Color Chart, 6th ed. RHS, London,
645 UK.
646
647 Rossi RJ. 2022. Applied Biostatistics for the Health Sciences. Wiley.
648 Salma U, Kundu S, Mandal N. 2017. Artificial polyploidy in medicinal plants: advancement in
649 the last two decades and impending prospects. *Journal of Crop Science and Biotechnology*, 20:
650 9-19. DOI: 10.1007/s12892-016-0080-1.
651
652 Samatadze TE, Yurkevich OY, Khazieva FM, Basalaeva IV, Konyaeva EA, Burova AE,
653 Zoshchuk SA, Morozov AI, Amosova AV, Muravenko OV. 2022. Agro-Morphological and
654 Cytogenetic Characterization of Colchicine-Induced Tetraploid Plants of *Polemonium caeruleum*
655 *L*. (*Polemoniaceae*). *Plants*, 11(19):2585. DOI:10.3390/plants11192585.
656
657 Sanders RW. 1987. Taxonomy of *Agastache* section Brittonastrum (Lamiaceae-Nepeteae).
658 *Systematic Botany Monographs*, 15:1-92. DOI: 10.2307/25027677.
659
660 Talebi SF, Saharkhiz MJ, Kermani MJ, Sharafi Y. 2021. Polyploidy increases tolerance to salt
661 stress in Anise hyssop (*Agastache foeniculum* [Pursh.] Kuntze). *Caryologia*, *74*(1): 33-41. DOI:
662 10.36253/caryologia-783.
663 Talebi SF, Saharkhiz MJ, Kermani MJ, Sharafi Y, Fard FR. 2016. Effect of ploidy level on the
664 nuclear genome content and essential oil composition of Anise Hyssop (*Agastache foeniculum*
665 [Pursh.] *Kuntze*). *Analytical Chemistry Letters*, 6(5): 678-687.
666 DOI: 10.1080/22297928.2016.1257953.
667
668 Talebi SF, Saharkhiz MJ, Kermani MJ, Sharafi Y, Fard FR. 2017. Effect of different antimitotic
669 agents on polyploid induction of anise hyssop (*Agastache foeniculum L.*). *Caryologia*, 70(2):
670 184-193. DOI: 10.1080/00087114.2017.1318502.
671
672 Tavan M, Azizi A, Sarikhani H, Mirjalili MH, Rigano MM. 2022. Induced polyploidy and broad
673 variation in phytochemical traits and altered gene expression in *Salvia multicaulis*. *Scientia*
674 *Horticulturae*, 291: 110592. DOI: 10.1016/j.scienta.2021.110592.
675

Tavan M, Mirjalili MH, Karimzadeh G. 2015. *In vitro* polyploidy induction: changes in morphological, anatomical and phytochemical characteristics of *Thymus persicus* (Lamiaceae). *Plant Cell Tissue Organ Culture,* 122: 573–583. DOI: 10.1007/s11240-015-0789-0.

Temel A, Gozukirmizi N. 2015. Cytotoxic effects of metaphase-arresting methods in barley. *Cytology and Genetics*, 49: 382-387. DOI: 10.3103/S0095452715060109.

Tsukaya, H. 2008. Controlling size in multicellular organs: focus on the leaf. *PLoS Biology*, 6(7): e174. DOI: 10.1371/journal.pbio.0060174.

Tulay E, Unal M. 2010. Production of colchicine induced tetraploids in *Vicia villosa* roth. *Caryologia*, 63(3): 292-303. DOI: 10.1080/00087114.2010.10589739.

Wickham H. 2016. ggplot2: elegant graphics for data analysis. *Springer*, New York. DOI: 10.1007/978-3-319-24277-4_9.

Yavari S, Omidbaigi R, Hassani ME. 2011. The effects of induction of autotetraploidy on the essential oil content and compositions of Dragonhead (*Dracocephalum moldavica* L.). *Journal of Essential Oil Bearing Plants*, 14(4): 484-489. DOI: 10.1080/0972060X.2011.10643605.

Yu Z, Haage K, Streit VE, Gierl A, Torres-Ruiz RA. 2009. A large number of tetraploid *Arabidopsis thaliana* lines, generated by a rapid strategy, reveal high stability of neo-tetraploids during consecutive generations. *Theoretical and Applied Genetics*, 118: 1107–1119. DOI: 10.1007/s00122-009-0966-9.

Urwin NA. 2014. Generation and characterization of colchicine-induced polyploid *Lavandula* × *intermedia*. *Euphytica*, 197: 331-339. DOI: 10.1007/s10681-014-1069-5.

Urwin NA, Horsnell J, Moon T. 2007. Generation and characterization of colchicine-induced autotetraploid *Lavandula angustifolia*. *Euphytica*, 156: 257-266. DOI: 10.1007/s10681-007-9373-y.

Zarate-Salazar JR, de Souza LM, de Morais MB, Neto LP, Willadino L, Gouveia-Neto A, Ulisses C. 2020. Light-emitting diodes and gas exchange facilitation minimize hyperhydricity in *Lippia grata*: Physiological, biochemical and morpho anatomical aspects. *South African Journal of Botany*, 135: 164-171. DOI: 10.1016/j.sajb.2020.08.019.

Zhang Z, Tan M, Zhang Y, Jia Y, Zhu S, Wang J, Zhao J, Liao Y, Xiang Z. 2021. Integrative analyses of targeted metabolome and transcriptome of *Isatidis Radix* autotetraploids highlighted

715 key polyploidization-responsive regulators. *BMC Genomics*, 22: 1-13. DOI: 10.1186/s12864-
716 021-07980-w.
717
718 Zhou K, Fleet P, Nevo E, Zhang X, Sun G. 2017. Transcriptome analysis reveals plant response
719 to colchicine treatment during on chromosome doubling. *Scientific Reports*, 7(1): 8503. DOI:
720 10.1038/s41598-017-08391-2.

Figure 1

Comparison of the leaf and stomata characteristics between diploid and induced tetraploid plants. Diploid (a,c) and tetraploid plant (b,d).

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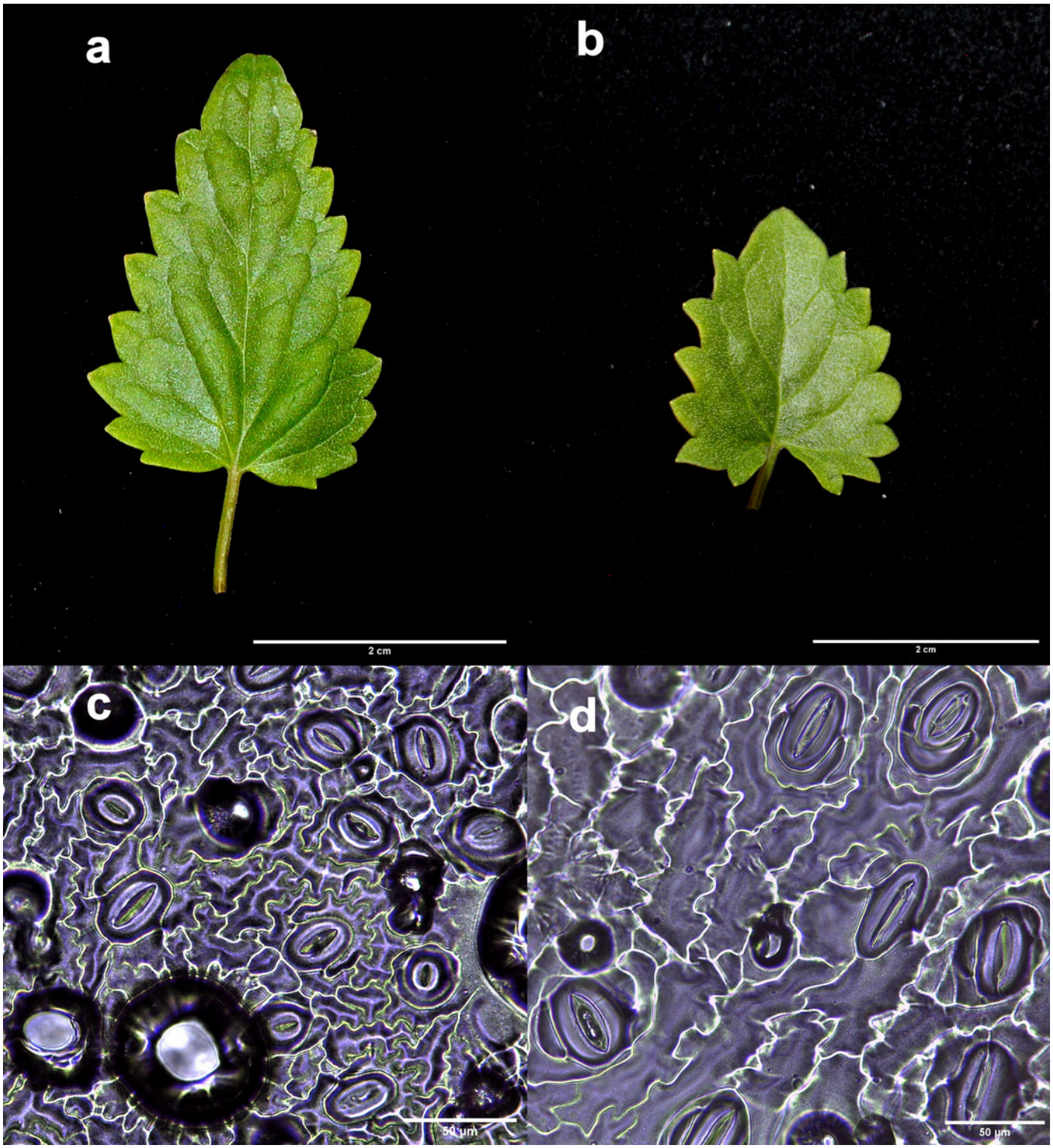
a
b
2 cm
2 cm
c
d
50 µm
50 µm

Figure 2

Chromosome counts of *A. mexicana*

(A) Diploid cell with 18 chromosomes and (B) tetraploid cell of *A. mexicana* with 36 chromosomes.

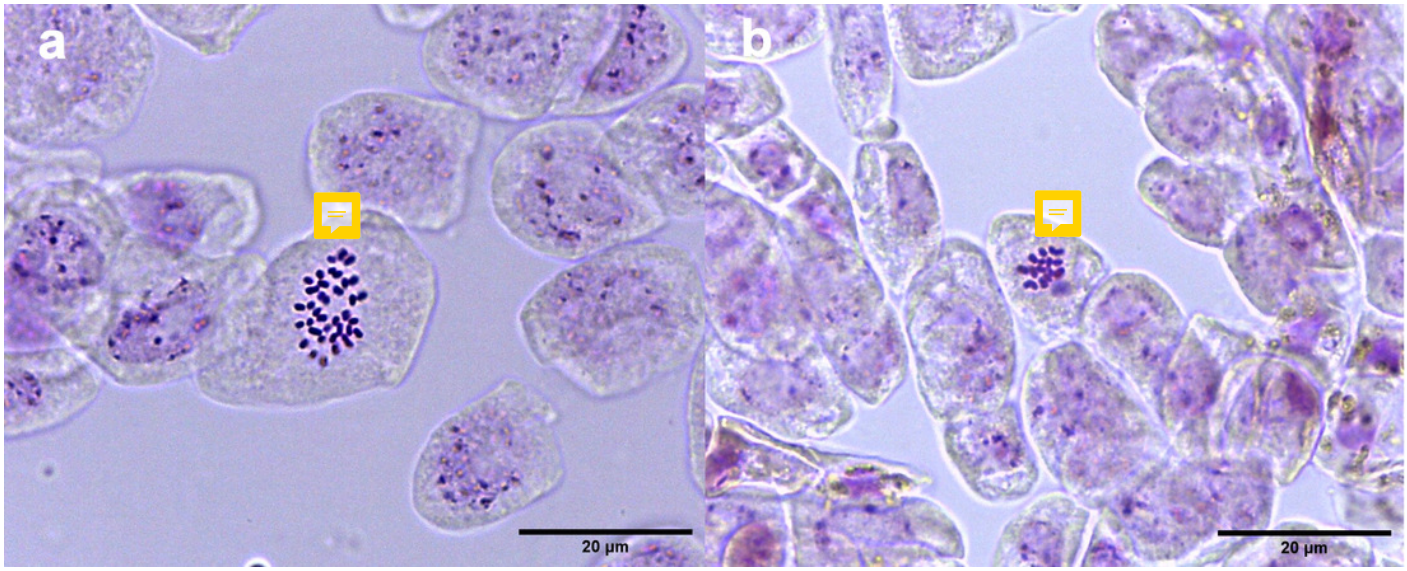

Figure 3

Flow cytometry analysis of *A. mexicana* (a) diploid and (b) induced tetraploid plant.

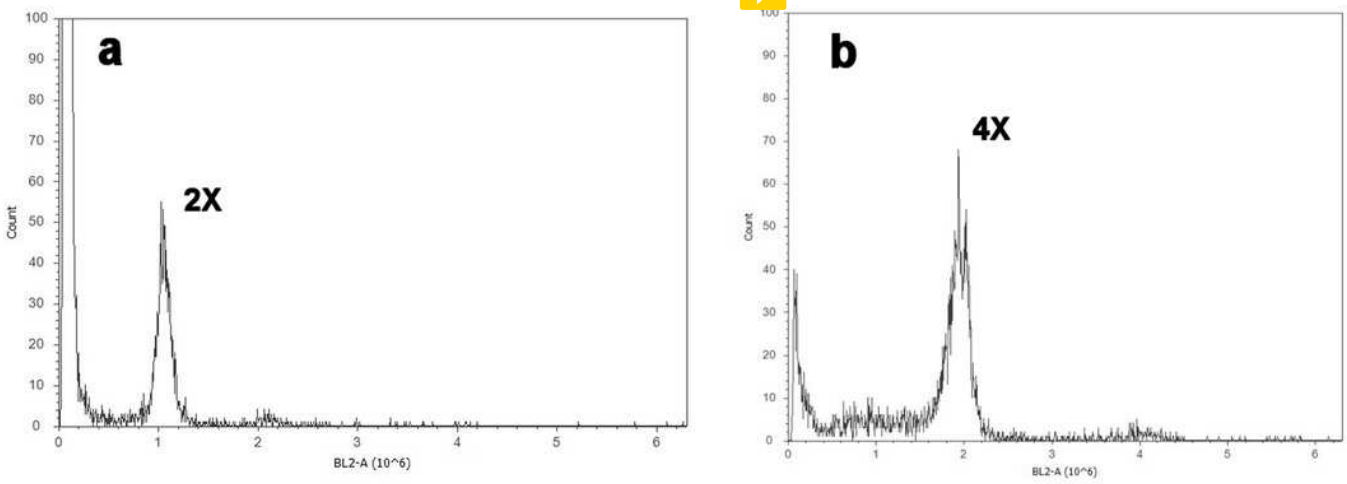

Figure 4

Total tilianin content in control and induced polyploidy (A*mx* 1-10) lines of *A. mexicana*.

Different letters on the vertical bars differ significantly (Tukey HSD Test, $P < 0.05$).

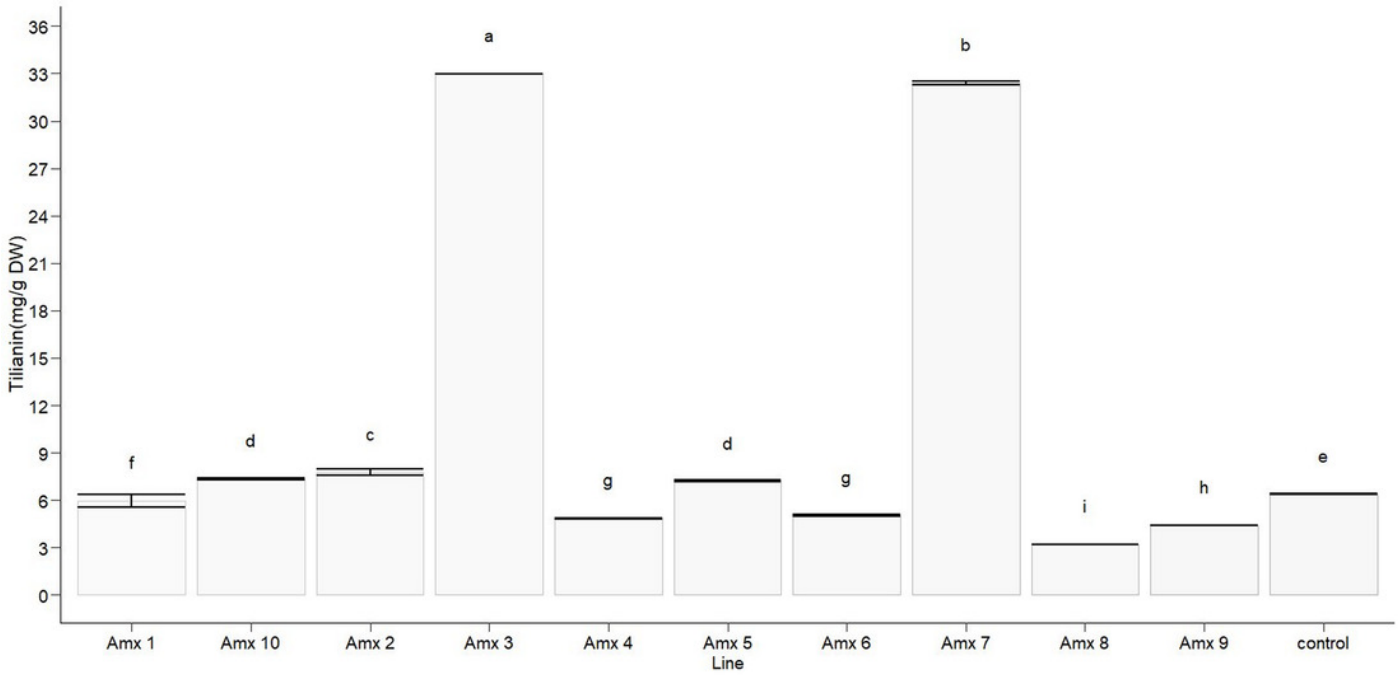

Figure 5

Principal component analysis of tetraploid and diploid control of *A. mexicana.*

a) Separation of the variables as a function of tetraploid and control lines. b) PC1 vs. PC2, with 57.05% variance. DNA; tilianin; Chlo T, total chlorophyll; LA, leaf area; FL, flower length; CL, calyx length; L, lightness; C*, chromaticity; h, hue angle.

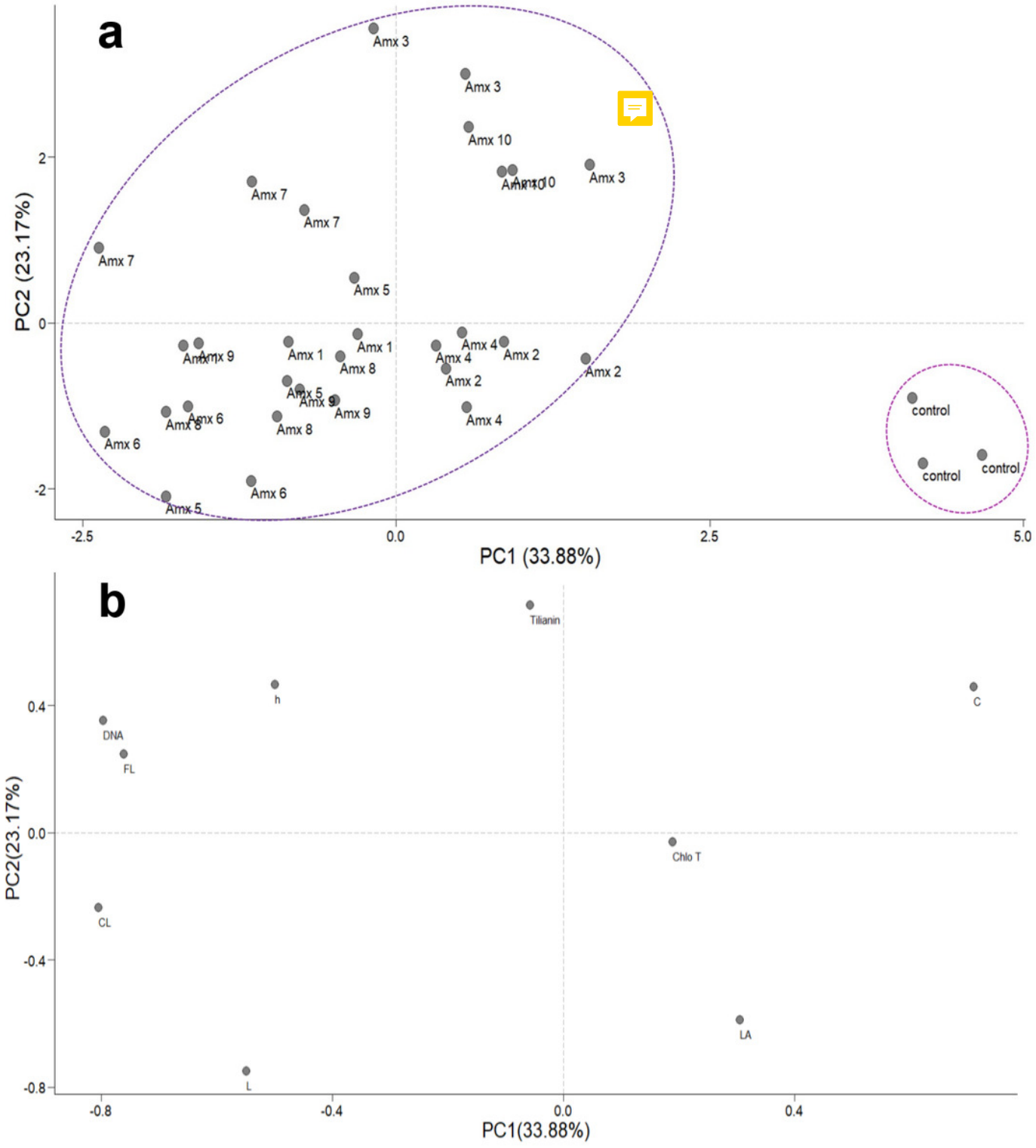
a
Amx 3
Amx 3
Amx 10
Amx 3
Amx 7
Amx 7
Amx 7
Amx 5
Amx 9
Amx 1
Amx 1
Amx 8
Amx 4
Amx 4
Amx 2
Amx 2
Amx 5
Amx 9
Amx 9
Amx 6
Amx 8
Amx 8
Amx 4
Amx 6
Amx 6
Amx 5
control
control
control
PC2 (23.17%)
PC1 (33.88%)
2
0
-2
-2.5
0.0
2.5
5.0
b
Tilianin
h
C
DNA
FL
Chlo T
CL
LA
L
PC2(23.17%)
PC1(33.88%)
0.4
0.0
-0.4
-0.8
-0.8
-0.4
0.0
0.4

Table 1(on next page)

Survival rate and polyploidy level of *Agastache mexicana* lines obtained after polyploidization treatments.

* Efficiency of polyploidization. Twelve seedlings were used per treatment.

Treatment		Survival rate (%)	Number of regenerated plantlets (Lines)	Ploidy level determined by flow cytometry		
Colchicine %	Time (h)			No. of diploid lines	No. of tetraploid lines (%)*	No. of mixoploid lines (%)*
0	6	100	19	19	0	0
0	12	100	19	19	0	0
0	24	100	21	21	0	0
0.1	6	58.33	12	1	11 (91.66)	0
0.1	12	50.00	7	2	4 (57.14)	1 (14.28)
0.1	24	8.33	7	1	6 (85.71)	0
0.3	6	8.33	0	0	0	0
0.3	12	0	0	0	0	0
0.3	24	16.66	0	0	0	0
0.5	6	8.33	3	1	2 (66.66)	0
0.5	12	8.33	0	0	0	0
0.5	24	8.33	0	0	0	0

1

Table 2(on next page)

Petal color and flower size of 10 tetraploid lines and a diploid control grown in the greenhouse.

Different letters indicate significant statistical differences between samples, defined by one-way ANOVA followed by Tukey's test for multiple comparisons at $p < 0.05$, with results referring to the mean of three observations ± SD.

Line	Flower length	Calyx length	Petal color			
			*L**	*C**	*h*	View
Control	25.252 ± 2.947 ^{d}	11.271 ± 2.334 ^{cd}	45.735 ± 2.831 ^{d}	32.336 ± 1.909 ^{a}	319.822 ± 1.481 ^{b}	
A*mx* 1	29.967 ± 1.759 ^{bc}	11.294 ± 0.596 ^{cd}	50.962 ± 2.356 ^{ab}	26.864 ± 2.154^{d}	321.572 ± 2.616 ^{ab}	
A*mx* 2	25.216 ± 3.599 ^{d}	10.624 ± 0.925 ^{d}	50.224 ± 4.043 ^{ab}	27.472 ± 4.371 ^{d}	323.039 ± 1.806 ^{a}	
A*mx* 3	30.842 ± 2.588 ^{bc}	11.092 ± 0.716 ^{cd}	46.126 ± 1.661 ^{d}	31.033 ±1.745 ^{ab}	323.238 ± 1.291 ^{a}	
A*mx* 4	30.555 ± 3.873 ^{bc}	11.486 ± 0.819 ^{cd}	49.639 ± 4.141 ^{bc}	30.579 ± 2.877 ^{abc}	322. 366 ± 2.870 ^{ab}	
A*mx* 5	31.622 ± 1.987 ^{ab}	12.609 ± 0.484 ^{ab}	51.238 ± 2.573 ^{ab}	28.915 ± 1.765 ^{bcd}	323.933 ± 1.258 ^{a}	
A*mx* 6	31.006 ± 2.631 ^{bc}	13.347 ± 0.843 ^{a}	50.878 ± 3.685 ^{ab}	27.984 ± 2.133 ^{dc}	322.306 ± 2.870 ^{ab}	
A*mx* 7	32.466 ± 0.832 ^{ab}	12.026 ± 1.048 ^{bc}	51.124 ± 3.532 ^{ab}	27.047 ± 3.421 ^{d}	322.85 ± 1.657 ^{a}	
A*mx* 8	34.094 ± 1.598 ^{a}	11.894 ± 0.449 ^{bc}	52.871 ± 1.451 ^{a}	26.573 ± 2.291 ^{d}	324.094 ± 6.688 ^{a}	
A*mx* 9	32.076 ± 3.609 ^{ab}	11.842 ± 0.697 ^{bc}	51.519 ± 2.175 ^{ab}	28.642 ± 1.4 ^{bcd}	321.483 ± 1.637 ^{ab}	
A*mx* 10	28.751 ± 2.134 ^{c}	11.113 ± 0.553 ^{cd}	46.721 ± 1.559 ^{dc}	31.182 ± 1.321 ^{ab}	324.006 ± 0.803 ^{a}	
ANOVA	$F_{10, 187}$= 19.89, P<0.0001	$F_{10, 187}$= 10.86, P<0.0001	$F_{10, 187}$= 12.59, P<0.0001	$F_{10, 187}$= 11.89, P<0.0001	$F_{10, 187}$= 3.993, P<0.0001	

1

Table 3(on next page)

Leaf area and chlorophyll content in mature leaves from 10 tetraploid lines and a diploid control grown in the greenhouse.

1

Line	Leaf area	Total chlorophyll	Chlorophyll a	Chlorophyll b
Control	8.561 ± 1.433 ^{ab}	363.7 ± 35.78^{bc}	278.6 ± 22.657 ^{bc}	85.1 ± 15.807 ^{b}
A*mx* 1	8.596 ± 3.999 ^{ab}	363.5 ± 21.072 ^{bc}	271.8 ± 26.317 ^{bc}	91.7 ± 12.074 ^{ab}
A*mx* 2	9.32 ± 3.377 ^{a}	414.1 ± 24.269 ^{ab}	296.1 ± 25.339 ^{abc}	118.0 ± 13.241 ^{a}
A*mx* 3	4.152 ± 1.413 ^{d}	390.8 ± 87.152 ^{abc}	297.9 ± 47.082 ^{abc}	92.9 ± 43.193 ^{ab}
A*mx* 4	8.205 ± 1.548 ^{abd}	345.6 ± 29.349 ^{c}	258.6 ± 22.061 ^{c}	87.0 ± 9.428 ^{b}
A*mx* 5	8.282 ± 3.465 ^{abd}	391.9 ± 38.155 ^{abc}	295.9 ± 30.555 ^{abc}	95.8 ± 11.849 ^{ab}
A*mx* 6	5.712 ± 1.238 ^{bcd}	421.8 ± 39.202 ^{ab}	319.2 ± 35.266 ^{ab}	102.6 ± 9.901 ^{ab}
A*mx* 7	4.570 ± 1.307 ^{d}	443.5 ± 73.971 ^{a}	333.4 ± 70.298 ^{a}	110.1 ± 15.058 ^{ab}
A*mx* 8	10.833 ± 3.066 ^{a}	383.7 ± 21.638 ^{abc}	294.1 ± 11.551 ^{abc}	89.6 ± 11.568 ^{ab}
A*mx* 9	8.675 ± 2.921 ^{ab}	385.6 ± 33.731 ^{abc}	296.4 ± 21.593 ^{abc}	89.2 ± 12.951 ^{ab}
A*mx* 10	4.93 ± 1.216 ^{cd}	411.5 ± 49.365^{abc}	314.6 ± 23.324 ^{ab}	96.9 ± 34.027 ^{ab}
ANOVA	$F_{10, 115} = 8.793$, $p < 0.0001$	$F_{10, 99} = 3.915$, $p < 0.001$	$F_{10, 99} = 3.929$, $p < 0.001$	$F_{10, 99} = 2.575$, $p < 0.01$

2