



## A protocol for the *in vitro* propagation and polyploidization of an interspecific hybrid of *Glandularia* (*G. peruviana* × *G. scrobiculata*)



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### ABSTRACT

The genus *Glandularia* (Verbenaceae) has more than 50 species and it holds great ornamental potential due to its colorful flowers, long flowering period and low water requirements. Since the floriculture market avidly seeks novelties, increasing the diversity within the genus would increase its commercial value. Some of the traits that should be improved in these species are their architecture and the size and colors of the flowers and leaves. In this sense, obtaining polyploid individuals is an interesting strategy to achieve this objective. In this paper a hybrid of *Glandularia peruviana* × *Glandularia scrobiculata* was cultured under *in vitro* conditions. Different plant growth regulators and their combinations were tested to obtain appropriate multiplication rates. The best results were obtained with the combination of 6.6 μM thidiazuron/0.03 μM α-naphthalenetic acid, with a multiplication rate of 19 shoots per explant. The plantlets were first rooted and then acclimatized by transferal to an 8.0 cm diameter pot containing Growing mix®, and maintenance inside a humidity chamber. For polyploidization, the explants were exposed to colchicine in concentrations of 0.001% and 0.01% for 24 and 48 h. The 40 recovered plants were characterized according to their DNA content. There were 21 diploids, 14 solid tetraploids, 1 solid octoploid, 3 chimera tetraploids and 1 chimera octoploid. Phenotypically, the size of the flowers, inflorescences, pollen grains and stomata were significantly larger in polyploid individuals. Surprisingly, stem diameter, leaf size and color intensity of the leaves and flowers were not significantly different between diploid and tetraploid individuals. Due to the size of the inflorescences, the tetraploid individuals are a promising starting material for a *Glandularia* breeding program.

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### 1. Introduction

The genus *Glandularia* (Verbenaceae), comprises approximately 50 species of herbaceous annual and perennial plants (Zuloaga and Morrone, 1999). These species are found in temperate and subtropical regions of North and South America. In Argentina, they are common in the provinces of Buenos Aires, Entre Ríos, Corrientes, Formosa and Santa Fe (Peralta and Mulgura, 2011). *Glandularia* has important ornamental potential due, mainly, to its

attractive flowers, which come in a wide variety of colors (white, red, pink, lilac or violet). Its long blooming period and low water requirements are also important features. Some of the members of this genus are erect, while others have creeping growth habits (Zuloaga and Morrone, 1999). Since the floriculture market avidly seeks novelties, increasing the diversity within the genus would increase its commercial value (Heywood, 2003).

Several objectives can be achieved when native plant germplasm with ornamental potential is used sustainably. For instance, it can provide the floriculture market with the novelties it so avidly seeks (Chandler and Tanaka, 2007; Rout et al., 2006). In this context, biotechnology offers a wide range of powerful tools for germplasm development. Plant tissue culture is based on the totipotency of plant cells and is one of the main tools employed in the floriculture industry because it allows the massive propagation of the selected species. Furthermore, it can also yield valuable insights into the physiology of the species. It is also the starting

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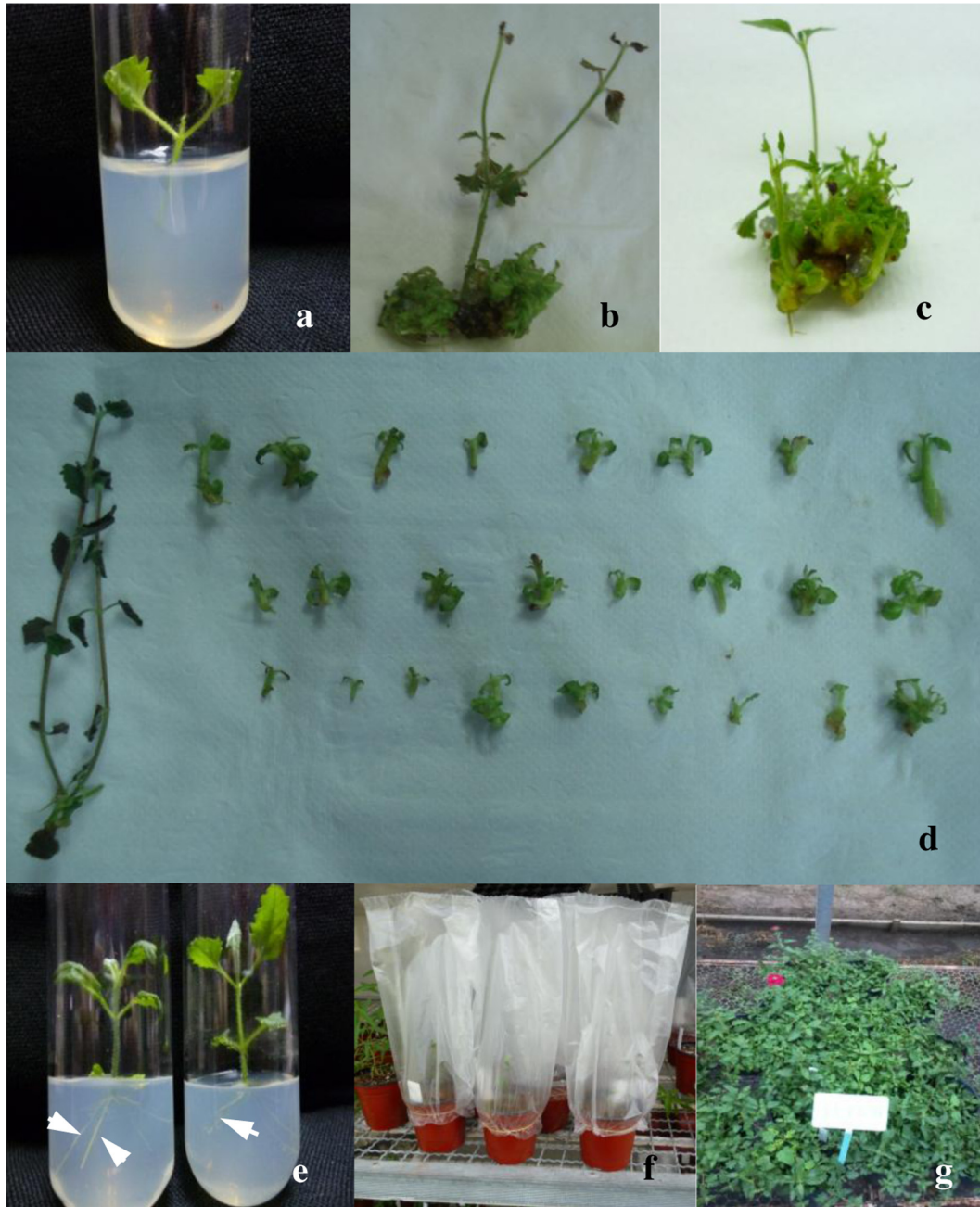
point for the application of other biotechnological techniques that can contribute to plant breeding (Escandón et al., 2010).

There are few reports regarding the application of biotechnological strategies for germplasm breeding of *Glandularia*, and this highlights the relevance of this research. In fact, the only references found regarding the *in vitro* propagation of this genus were the previous works of this group on *Glandularia peruviana* (Iannicelli et al., 2010a,b; 2012) and the research carried out by Marino et al. (2003) and Ponce et al. (2010).

Polyploidization has played a leading role in the evolutionary development of many plant species (Chen and Zhongfu, 2006). In recent years, synthetic ploidy breeding has also made an important contribution to crop domestication and to the development of

new cultivars, e.g., creating crops with specific traits such as larger flowers and fruits (Yang et al., 2011). This methodology has been historically used, especially in ornamental crops, to obtain new morphological characteristics or to overcome interspecific crossing barriers (Eckhaut et al., 2006; Horn, 2002). However, there is still limited knowledge concerning all the effects that polyploidization can cause in plant processes. Obtaining autopolyploid individuals by application of the mutagenic agent colchicine increases the variability of phenotypes by altering the size, shape and color of leaves, flowers and stems and it can also affect plant architecture (Eckhaut et al., 2004; Escandón et al., 2005, 2006, 2007; Dhooghe et al., 2011).

In this paper, we report a protocol to obtain new phenotypes of *Glandularia* from an artificial hybrid between *G. peruviana* (Griseb)



**Fig. 1.** *In vitro* development of *Glandularia* hybrid. The explants were single-node segments. (a) Early stage of *in vitro* culture. (b) Explant cultured on WPM + 6.6  $\mu$ M TDZ (c) Explant cultured on WPM + 6.6  $\mu$ M TDZ/0.03  $\mu$ M NAA. In both (b and c), shoot development from the base of the explant was observed after 30 days. (d) 25 shoots recovered from one explant cultured on WPM + 6.6  $\mu$ M TDZ/0.03  $\mu$ M NAA. (e) Plantlets during de-rooting step (roots arrowed). (f) Acclimatization phase. (g) Viable *ex vitro* plants growing under standard greenhouse conditions.

and *Glandularia scrobiculata* Tronc. (Stancanelli et al., unpublished), based on the combination of tissue culture techniques and colchicine-induced polyploidization. Hypothetically, this approach could significantly amplify genotypic and phenotypic variability.

## 2. Materials and methods

### 2.1. Explant disinfection and establishment and multiplication of *Glandularia* hybrid in vitro cultures

Nodal segments and apices obtained from a *G. peruviana* × *G. scrobiculata* hybrid were used as explants. The donor plant was grown in standard greenhouse conditions and, prior to

explanting, was sprayed three times every two days with 2.5 ml l<sup>-1</sup> of Kasumin® (Kasugamycine: fungicide and bactericide).

According to the protocol described in Iannicelli et al. (2012), to disinfect the explants, 1.0 cm long nodal segments and apices were washed with tap water for 10 min, followed by sonication for 20 min. Afterwards, the explants were submerged in ethanol 70% (1 min), followed by 0.55% sodium hypochlorite (20 min) and 0.01% Tween 80. Finally, in a laminar flow cabinet, they were washed 3 times with sterile distilled water and sowed on woody plant medium (WPM) (McCown and Lloyd, 1981) without plant growth regulators (PGR), supplemented with 20 g l<sup>-1</sup> sucrose and solidified with 0.7% agar (Sigma®). The medium pH was adjusted to 5.6 with KOH (0.1 M and 0.5 M) and HCl (0.1 M and 0.5 M) and autoclaved 17 min at 121 °C and 1013 bar.

**Table 1**  
Multiplication rate (average number of shoots per explant) observed after 30 days of culture with different concentrations of TDZ combined with or without NAA. Different letters indicate differences between treatments (Tukey test,  $p < 0.05$ ). n/d: not determined.

		TDZ (μM)						
NAA (μM)	0.0	0.0	0.4	1.1	2.2	4.5	6.6	9.0
	0.0	1 ± 0.40a	1 ± 0.75a	1 ± 0.80a	3 ± 1.26a	2 ± 0.90a	6 ± 1.41b	3 ± 1.31a
	0.3	n/d	n/d	n/d	n/d	n/d	19 ± 2.62c	n/d
	0.6	n/d	n/d	n/d	n/d	n/d	5 ± 1.06b	n/d

**Table 2**  
Comparison of DNA amounts and flower diameter among the plants recovered from the colchicine assay. Treatment 1: 0.001%/colchicine/24 h. Treatment 2: 0.001%/colchicine/48 h. Treatment 3: 0.01% colchicine/24 h. Treatment 4: 0.01% colchicine/48 h. Mean ± SD. Different letters indicate differences between treatments (Tukey  $p < 0.05$ ). Polyploid plants in bold. C: chimera.

	Individuals	DNA amount	Ploidy level	Flower diameter (mm)
	Donor plant	18.96a	2n	11.80 ± 0.24a
	Average controls	17.02a	2n	11.46 ± 0.17a
Individuals of treatment 1	1.11	16.24 ± 4.62a	2n	12.55 ± 0.60b
	1.12	16.19 ± 4.63a	2n	11.71 ± 0.18a
	1.14	16.65 ± 4.50a	2n	11.50 ± 0.22a
	1.17	15.98 ± 7.82a	2n	11.99 ± 0.14a
	1.18	15.45 ± 4.86a	2n	12.33 ± 0.13b
	<b>1.13</b>	<b>36.74 ± 4.21b</b>	<b>4n</b>	13.72 ± 0.16d
	<b>1.16</b>	<b>34.22 ± 4.72b</b>	<b>4n</b>	13.70 ± 0.24d
Individuals of treatment 2	2.1	15.97 ± 4.86a	2n	12.16 ± 0.25b
	2.4	15.33 ± 4.89a	2n	12.21 ± 0.22b
	2.5	15.53 ± 4.83a	2n	11.20 ± 0.24a
	2.10	17.32 ± 7.22a	2n	12.20 ± 0.11b
	<b>2.2</b>	<b>34.25 ± 6.57b</b>	<b>4n</b>	13.63 ± 0.22d
	<b>2.3</b>	<b>30.16 ± 2.49b</b>	<b>4n</b>	13.88 ± 0.11e
	<b>2.6</b>	<b>35.17 ± 2.13b</b>	<b>4n</b>	13.5 ± 0.35d
	<b>2.9</b>	<b>34.5 ± 3.62b</b>	<b>4n</b>	13.37 ± 0.36d
	<b>2.7</b>	<b>62.7 ± 8.37c</b>	<b>8n</b>	13.9 ± 0.10e
	<b>2.8</b>	<b>30.85 ± 2.43b–62.91 ± 3.58c</b>	<b>C</b>	14.02 ± 0.09e
Individuals of treatment 3	3.1	15.52 ± 8.00a	2n	11.50 ± 0.06a
	3.2	14.93 ± 5.02a	2n	12.78 ± 0.26b
	3.4	16.08 ± 4.66a	2n	10.10 ± 0.55a
	3.5	15.96 ± 4.70a	2n	12.28 ± 0.29b
	3.6	15.54 ± 4.83a	2n	11.20 ± 0.11a
	3.7	18.62 ± 4.08a	2n	12.53 ± 0.08b
	3.8	15.54 ± 4.66a	2n	12.17 ± 0.14b
	3.9	16.86 ± 7.42a	2n	12.01 ± 0.11b
	3.13	15.97 ± 4.70a	2n	12.04 ± 0.08b
	3.14	15.51 ± 8.06a	2n	10.97 ± 0.07a
	<b>3.11</b>	<b>14.8 ± 5.23a–30.8 ± 4.06b</b>	<b>C</b>	13.03 ± 0.13c
	<b>3.12</b>	<b>37.15 ± 4.71b</b>	<b>4n</b>	13.81 ± 0.09e
	Individuals of treatment 4	4.3	14.86 ± 1.68a	2n
4.6		15.70 ± 4.94a	2n	11.00 ± 0.23a
<b>4.1</b>		<b>30.02 ± 2.58b</b>	<b>4n</b>	13.10 ± 0.19d
<b>4.2</b>		<b>36.63 ± 3.41b</b>	<b>4n</b>	13.50 ± 0.19d
<b>4.4</b>		<b>30.83 ± 4.05b</b>	<b>4n</b>	13.20 ± 0.25d
<b>4.5</b>		<b>39.49 ± 5.70b</b>	<b>4n</b>	13.10 ± 0.19d
<b>4.10</b>		<b>34.73 ± 5.04b</b>	<b>4n</b>	13.70 ± 0.10e
<b>4.11</b>		<b>33.57 ± 3.72b</b>	<b>4n</b>	14.00 ± 0.08e
<b>4.13</b>		<b>37.65 ± 4.63b</b>	<b>4n</b>	14.00 ± 0.00e
<b>4.8</b>		<b>17.5 ± 7.97a–37.5 ± 8.68b</b>	<b>C</b>	13.6 ± 0.22d
<b>4.12</b>		<b>14 ± 5.28a–30.58 ± 4.09b</b>	<b>C</b>	14.00 ± 0.04e



After 48 h, the disinfection process was repeated and, after rinsing with sterile water, the oxidized sectors were removed and the explants were transferred to the same fresh medium. A total of 54 explants were used for the establishment of the *in vitro* culture of the *Glandularia* interspecific hybrid. Each one was grown in a 24 × 150 mm glass tube, containing 10 ml of WPM medium, covered with sterile gauze/cotton plugs. After 21 days, subcultures were performed on the same medium. Nodal segments, obtained from these plantlets cultured on PGR-free WPM, were transferred to the same basal medium but supplemented with different PGRs or combinations of PGRs. In all the assays reported in this paper, the physical culture conditions consisted of 16-h photoperiod using fluorescent tubes ( $52 \mu\text{mol} \times \text{m}^{-2} \times \text{s}^{-1}$ ) and a temperature of  $23 \pm 2^\circ\text{C}$ .

## 2.2. Culturing with thidiazuron (TDZ)

The following concentrations of the cytokinin TDZ were tested ( $\mu\text{M}$ ): 0.0; 0.4; 1.1; 2.2; 4.5; 6.6 and 9.0. There were eight explants per treatment and the experiment was replicated three times. After 30 days, the explants were subcultured to the same medium, but PGR-free, for the rooting step.

## 2.3. Culturing with a combination of $\alpha$ -naphthalenacetic acid (NAA)/TDZ

The following amounts of NAA ( $\mu\text{M}$ ): 0.0; 0.3 and 0.6 and TDZ ( $\mu\text{M}$ ) 0.0 and 6.6 were tested in all possible combinations. The explants were maintained under these conditions for 30 days and were then transferred to the same medium, but devoid of PGRs, for the rooting step. The experiment had three replicates with eight explants per treatment.

## 2.4. Rooting and acclimatization

Shoots that were three to four cm long were transferred to the rooting medium. The rooted plantlets were acclimatized as described by Escandón et al. (2003): they were transferred to an 8.0 cm diameter pot containing Growing mix<sup>®</sup>, and maintained inside a humidity chamber. The nylon bags used to make the humidity chamber were gently perforated every day until no condensation was detected inside them. The absence of condensation determined the end of the acclimatization stage. Afterwards, the plants were grown under standard greenhouse conditions.

## 2.5. *In vitro* plant polyploidization

Nodal segments of *Glandularia* hybrids were pre-cultured for 7 days on WPM supplemented with 0.3  $\mu\text{M}$  NAA and 6.6  $\mu\text{M}$  TDZ. Afterwards, they were treated with different doses of colchicine. The alkaloid solutions were prepared by dissolving colchicine in 1% dimethyl sulfoxide (DMSO) to obtain the following final concentrations (v/v): 0.0; 0.001 and 0.01%. Nodal segments were exposed for 24 and 48 h. The control treatments consisted of: (1) untreated nodal segments (2) nodal segments submerged in water; (3) nodal segments submerged in 1% DMSO (water solution). After the treatment, the explants were transferred to the same medium. The number of explants per treatment was 15.

To determine the ploidy level, the samples were analyzed with a flow cytometer (CyFlow Ploidy Analyser, Partec), and subsequently confirmed by chromosome counting. For the flow-cytometry measurements, and according to the manufacturer's instructions, approximately 0.5 cm<sup>2</sup> of leaf tissue taken from all the branches of the plant was submerged in a stripping buffer (Otto buffer I) consisting of 0.1 M citric acid and 0.5% Tween 20 (Otto, 1990) and chopped with a sharp razor blade. After filtration, the solution was stained

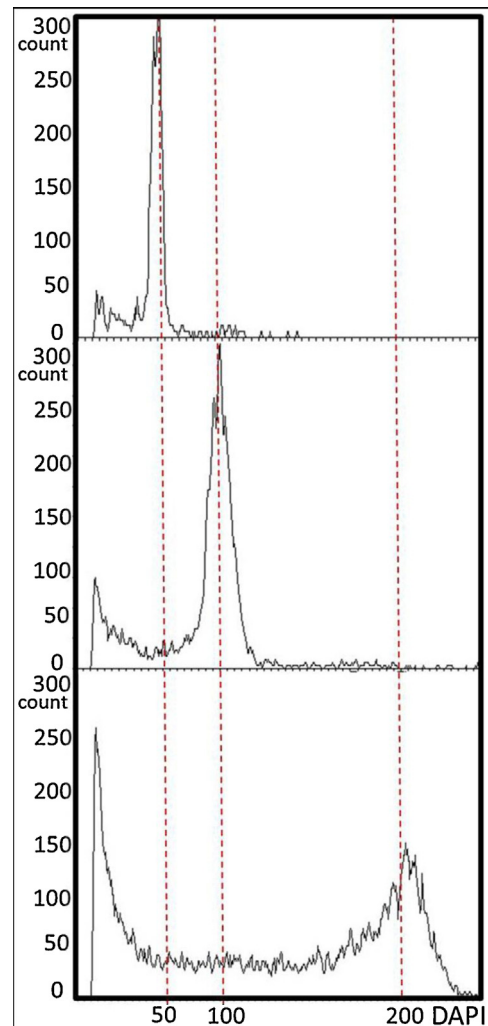
with a solution of 4,6-diamidino 2-phenyl-indole-dihydrochloride ( $4 \mu\text{g ml}^{-1}$ ) (DAPI) (Sigma D9542) in a buffer solution (Otto buffer II) composed of 0.4 M Na<sub>2</sub>HPO<sub>4</sub> (Otto, 1990). The different flow cytometer parameters were adjusted with non-treated material to obtain well defined and reproducible readings. For the diploid material the flow cytometer was adjusted to a Gain of 50. The nuclear DNA of 60 colchicine treated plants and their control were estimated by the fluorescence peaks obtained.

For chromosome counting, cuttings of diploid and tetraploid individuals were treated with indol-butyric acid (IBA) (0.01 M) and placed in a substrate composed of peat, perlite and vermiculite (2:1:1). Roots in active mitotic division were collected, pretreated with colchicine (0.025%) and fixed in Farmer solution (ethanol-acetic acid 3:1), Feulgen staining (Darlington and La Cour, 1962) after previous hydrolysis with HCl 1 M for 5 min at 60 °C, was performed. Once colored, roots were treated for 20 min with

**Table 3**

Comparison of stomata and pollen grain areas in plants with different ploidy level. Mean  $\pm$  SD "N": size of the samples. Different letters indicate significant differences (Tukey test  $p \leq 0.05$ ). n/d: not determined.

Ploidy level	N	Stomata area ( $\mu\text{m}^2$ )	N	Pollen grain area ( $\mu\text{m}^2$ )
2n	50	231.56 $\pm$ 70.00a	20	2092.99 $\pm$ 265.29a
4n	50	580.74 $\pm$ 62.00b	20	2878.85 $\pm$ 254.73b
8n	25	1380.89 $\pm$ 59.00c	n/d	n/d



**Fig. 2.** Profiles obtained by flow cytometry: Panel (a) diploid plant; panel (b) tetraploid plant; Panel (c) octoploid plant.

a buffer solution pH 4.5–4.8 (citric acid–sodium citrate, 0.01 M) at 37 °C, with an enzyme mixture of pectinase Sigma from *Aspergillus niger*, (1%) and cellulase Sigma from *A. niger* (2%). At least 10 metaphasic cells from each individual were analyzed under optical microscope.

### 2.6. Phenotypic characterization of recovered plants

The recovered plants (control and treated plants) were characterized by measuring the flower and stem diameter and the size of the leaves. The third leaf pair, counting from the apex, was chosen to measure both the stem diameter and the length  $\times$  width ratio to estimate the size of the leaves (leaf area index). In all cases, the average of 15 determinations was taken as a reference value.

In addition, the diameter of fifty stomata (which include both the pore itself and its accompanying guard cells) and twenty pollen grains of individuals of different ploidy levels were measured. To measure stomata, pieces of leaves were prepared in fresh and placed on slides with the abaxial side upturned and observed under microscope. Pollen grains were stained with Malachite green (0.1%) and mounted onto slides according to the protocol suggested by Alexander (1969). The measurement of stomata and pollen grains was conducted with the help of the software Cell sense, and a microscope Olympus model DP72, using an augmentation of 20 $\times$  for both stomata and pollen grain (Cell Sens Standar–Copyright© 2010. Olympus Corporation. ([www.olympus-sis.com](http://www.olympus-sis.com))). The color of leaves and flower was measured by a Minolta CR 321 colorimeter.

### 2.7. Statistical analysis

The experiments were conducted according to a complete randomized design. Data were statistically analyzed by analysis of variance (ANOVA) followed by Tukey test (99%) supported by the software Infostat E. version 2009e.

## 3. Results

### 3.1. Tissue culture

After the disinfection protocol, 70% of the treated explants were recovered. Only 30% were affected by the amount of ethanol and chlorine used, which was observed by browning and oxidation of the explants. The *in vitro* plantlets obtained were the source of the explants used in the rest of the experiments (Fig. 1a).

### 3.2. Culturing with different concentrations of TDZ

After one week of culture, internode elongation and adventitious root development were observed in the control explants. The same response was detected for the 0.4  $\mu$ M TDZ treatment, but with concentrations of TDZ above 1.1  $\mu$ M, there was a gradual reduction in the formation of adventitious roots, and an increase in the proliferation of morphogenetic calli. The most effective treatment (6.6  $\mu$ M TDZ) resulted in a callus that generated an average of 6 shoots per explant (Fig. 1b and Table 1) after 30 days of culture.

### 3.3. Culturing with combinations of NAA/TDZ

This resulted in fast induction of callus at the base of the explant followed by the development of shoots. After two weeks of culture, in all treatments containing the TDZ/NAA combinations, the explants showed a significant thickening at the base. After 30 days of culture, *de novo* buds developed from this morphogenetic callus (Fig. 1c), comparable to culturing with TDZ alone (6.6  $\mu$ M). The number of buds *per explant* obtained depended on the PGR ratio. The best response was obtained with 6.6  $\mu$ M TDZ/0.03  $\mu$ M NAA, with an average of 19 buds per explant (Fig. 1d, Table 1). Once the shoots were 2–3 cm long, they were transferred to the rooting medium, where all the treated plantlets developed adventitious roots. These plantlets were then subjected to acclimatization

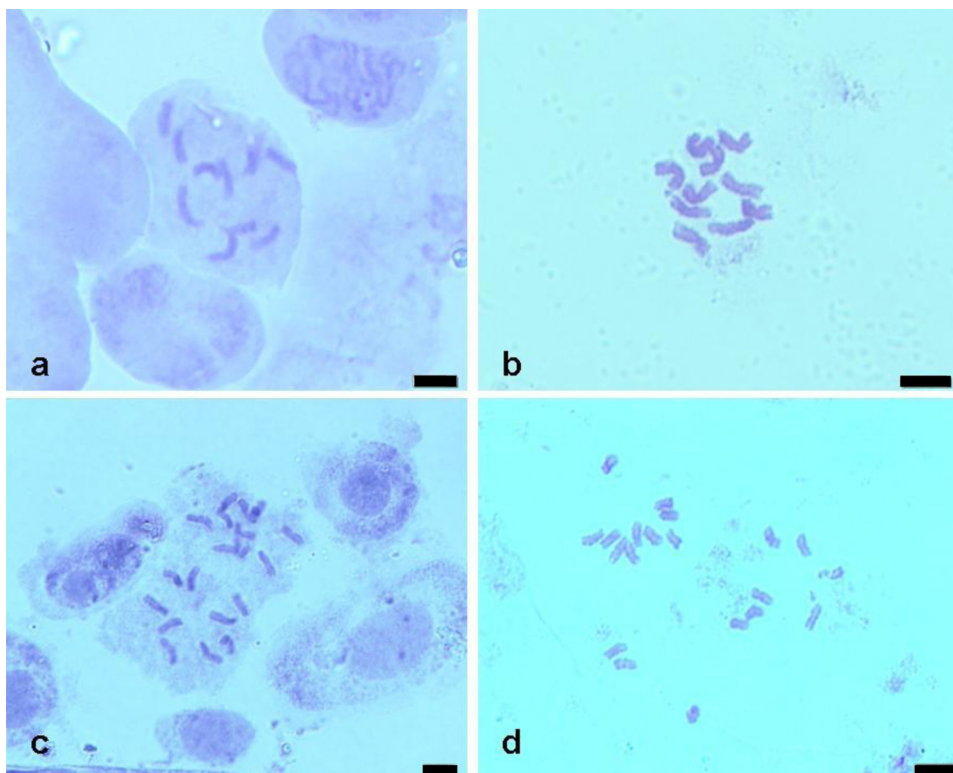
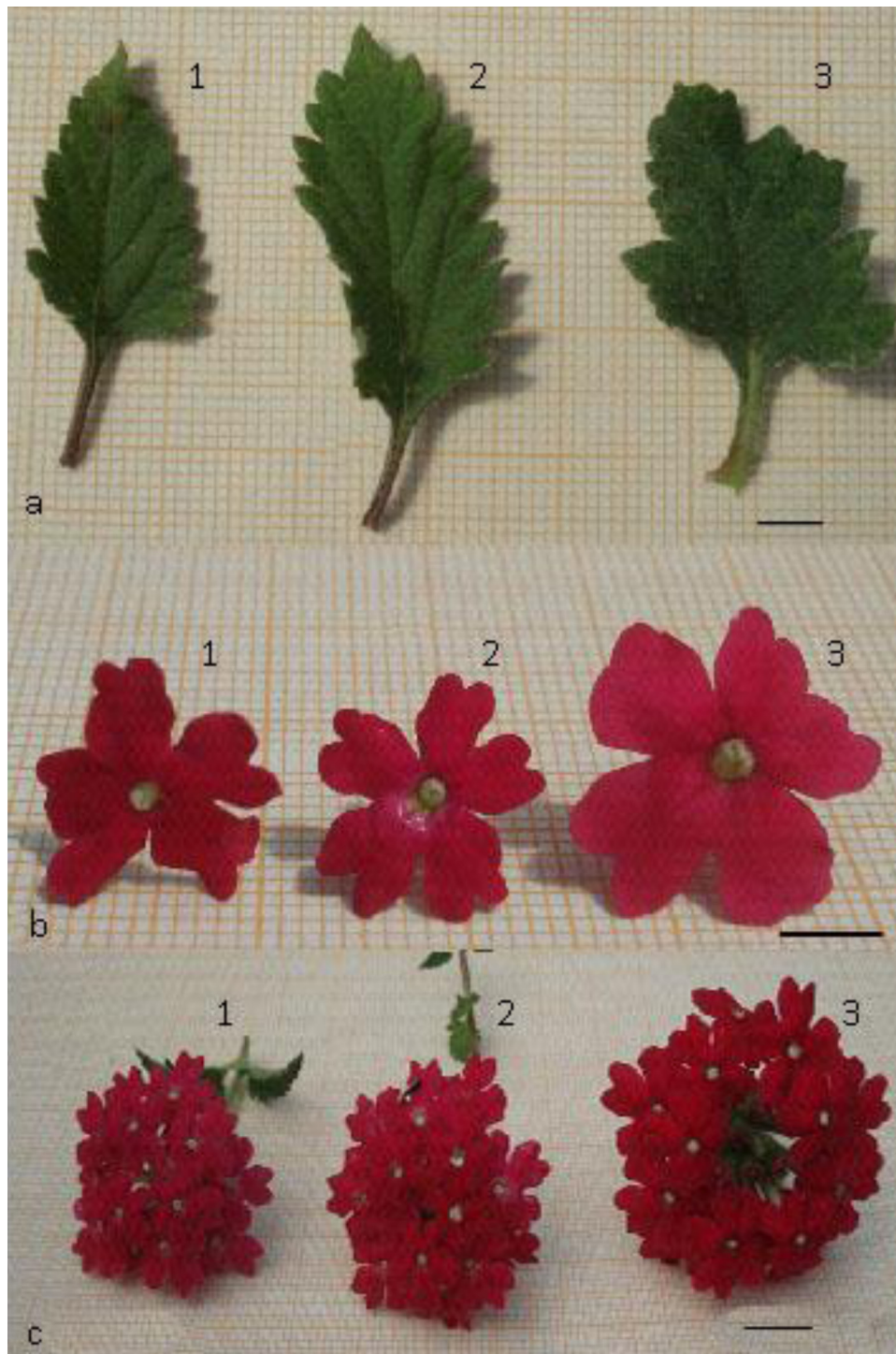


Fig. 3. Chromosome counting in *Glandularia* hybrid mitotic cells. (a and b) Diploid cytotypes ( $2n = 2x = 10$ ); (c and d) Tetraploid induced cytotypes ( $2n = 4x = 20$ ). Bars = 5  $\mu$ m.



**Fig. 4.** Comparison of leaves, flowers and inflorescences in diploid and polyploid individuals. Panel (a): (1) leaf of a diploid individual; (2) leaf of a tetraploid individual; (3) leaf of an octoploid individual. All leaves used as a sample were taken from the 4th whorl. Panel (b): (1 and 2) flowers of a diploid plants; (3) flower of a tetraploid plant. All the flowers were taken from fully developed inflorescences. Panel (c): (1) and (2) Inflorescences corresponding to diploid individuals (3) tetraploid inflorescence. In each panel: Bar: 1 cm.

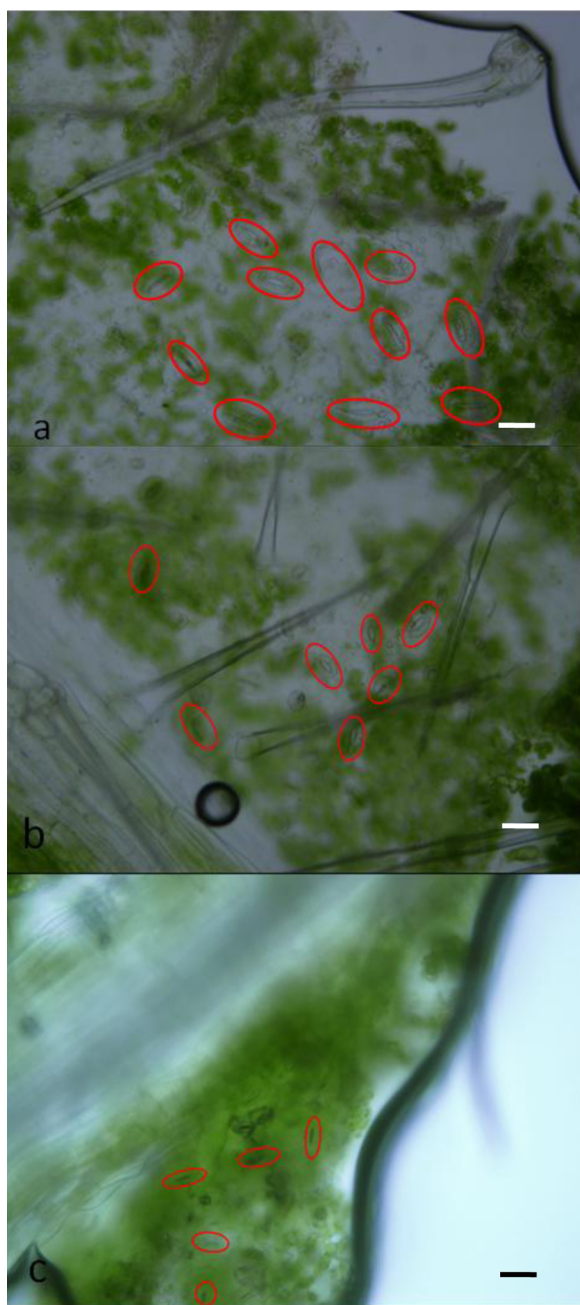
procedures (Fig. 1f). Finally, rooted and acclimatized plantlets were transferred and grown under greenhouse conditions. All the plants were shown to be viable (Fig. 1g).

### 3.4. Polyploidization

Table 2 compares the contents of DNA and the diameters of the flowers of the individuals recovered from the four colchicine

treatments, the controls and the mother plant. This Table shows the presence of polyploid individuals in all the colchicine treatments. In contrast, no polyploid individuals were detected in the control treatments. Regardless of the treatment, a total of 40 plants were recovered. Of those, 21 were diploid plants, 14 solid tetraploid plants, 1 solid octoploid plant, 3 chimera tetraploid plants and 1 chimera octoploid plant. Furthermore, it can be observed that the majority of polyploid plants recovered were obtained with both





**Fig. 5.** Comparison among the length of stomata of plant with different ploidy level. The red circles indicate the stomata of the leaves of plants recovered from the colchicine treatment. (a) octoploid plant. (b) tetraploid plant and (c) diploid plant (control). The difference in size of stomata was considered significant by the Tukey test. Bar: 20  $\mu$ m.

48-h treatments. Table 2 also shows that significant differences were detected in the floral diameter between diploid and polyploid individuals (see also Fig. 4b and c). On the other hand, the leaves size and the stem diameter showed a great variability and no differences between individuals of different ploidy levels could be detected (data not shown).

Fig. 2 exemplifies the graphics obtained for the DNA content of different individuals recovered from the colchicine treatment. Fig. 2a indicates the mean of relative DNA content of a diploid plant belonging to a control treatment, while Fig. 2b shows the peak for a tetraploid plant. In Fig. 2c, the peak corresponding to the octoploid individual can be observed.

Fig. 3a and b shows the chromosomes of two different genotypes of diploid plants. In Fig. 3c and d, different chromosomes from individuals treated with colchicines can be observed. The chromosomes counted were  $2n=10$  for diploid genotypes (Fig. 3a and b) and  $2n=20$  in the colchicine treated ones (Fig. 3c and d), thus confirming the effectiveness of induced polyploidization.

Fig. 4 shows leaves (Fig. 4a), flowers (Fig. 4b) and inflorescences (Fig. 4c) from colchicine treated plants with different ploidy levels. In Fig. 4a, leaf “1” corresponds to a diploid individual, leaf “2” to a tetraploid one and leaf “3” to a solid octoploid plant. In Fig. 4b, the flowers numbered “1” and “2” belong to a diploid individual, whereas number 3 is a tetraploid flower. Fig. 4c shows the correlation between the size of the flowers and the inflorescences. The inflorescence indicated as (1) is tetraploid; while inflorescences (2) and (3) are diploid.

Fig. 5 comparing the areas of stomata, which include both the pore itself and its accompanying guard cells, belongs to plants with different ploidy level; Table 3 confirms the differences observed in Fig. 5.

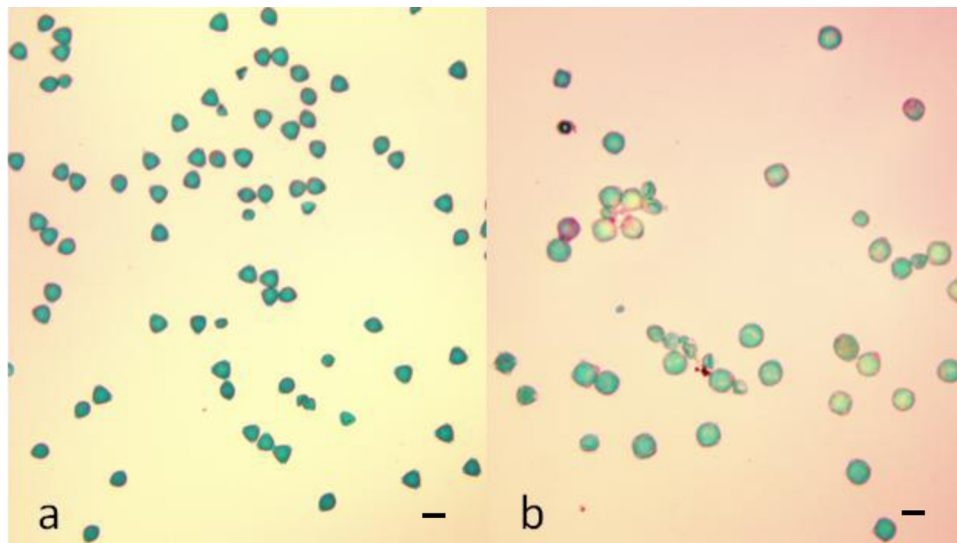
The size of pollen grain is another indicative parameter for polyploidy. Table 3 also indicates the difference sizes between the diploid pollen grains in relationship the tetraploid ones, this change can be clearly seen in Fig. 6.

The graphs in Fig. 7 compare the population points obtained by the colorimetric study of leaves (Fig. 7a) and flowers (Fig. 7b). In Fig. 7a, it can be observed that, among the individuals analyzed by the color of their leaves, three populations could be clearly distinguished: (1) the mother plant (brown dots), situated in the positive areas of the three coordinates; (2) the tetraploid individuals, designated as 4.1 (green points), which are set apart from the cloud where the majority of the dots are situated because they are located in positive values of “L” and (3) the remaining plants analyzed. The isolated orange and blue dots were the only exception.

The colorimetric study of the flowers (Fig. 7b), shows two distinct and clearly separate populations. One of them belongs to the mother plant (mustard points), situated in the positive areas of “L” and “b”. The other population corresponds to the rest of the individuals analyzed, which group together in the positive area of the “a” and “L” axes, indicating a clear tendency towards the color red in the flowers belonging to these plants.

#### 4. Discussion

Managing *in vitro* cultures of a particular species requires adequate knowledge concerning both its nutritional needs and the optimal combination of growth regulators. Previous studies in *Glandularia peruviana* (Iannicelli et al., 2012), *Gorytvesica tenera* (Iannicelli et al., 2010a) and *Glandularia* sp (Iannicelli et al., 2010b), indicated that WPM was the most suitable medium for the establishment of *in vitro* cultures of these species. Following the protocol proposed by Iannicelli et al. (2012) for *G. peruviana*, one of the parental species of the hybrid under study, the *in vitro* establishment of the *Glandularia* hybrid did not present major difficulties. This suggests that the nutritional requirements of this hybrid and those of the species studied in previous papers are similar (Iannicelli et al., 2010a,b; 2012). Based on the results obtained for *G. peruviana* by Iannicelli et al. (2012), the first experiments tested TDZ alone. However, when the medium was supplemented solely with this PGR, a multiplication rate of only 7 buds per explant was achieved. This could be considered a good result, since the regenerated shoots grew vigorously and were viable (data not shown). However, since the aim of this work was to obtain polyploid individuals *in vitro* through the



**Fig. 6.** (a) Grains of pollen of a diploid plant. (b) Grains of pollen of a tetraploid plant. Stained with the Alexander Technique. Magnification: 200 $\times$ . Bar: 100  $\mu$ m.

application of colchicine, the required multiplication rate was much higher (Escandón et al., 2007). In an attempt to increase it, a combination of a TDZ and NAA was tested. These combinations usually elicit diverse responses in different species (Rout et al., 2006). The best results were achieved with the combination 6.6  $\mu$ M TDZ/0.3  $\mu$ M NAA, in which 19 shoots per explant were obtained.

These results, compared to previously published ones, imply that different species of *Glandularia* have very different PGR requirements, despite belonging to the same genus. In fact, Iannicelli et al. (2012), while working with *G. peruviana*, reported a multiplication rate of more than 10 shoots per explant at low concentrations of TDZ. These authors also compared TDZ with other cytokinins and concluded that TDZ was the best cytokinin for that species. However, when TDZ was added to the culture medium of *G. tenera*, it caused oxidation and explant death (Iannicelli et al., 2010a). While in the present study the addition of an auxin was necessary to obtain a proper multiplication rate, Iannicelli et al. (2012) reported that, for *G. peruviana*, the combined use of an auxin and a cytokinin (BAP) induced the development of callus, roots and deformed buds (Iannicelli et al., 2012).

Colchicine caused a decrease in the growth rate of the explants and *plantlets in vitro*, possibly due to physiological disturbances (Yemet and Blume, 2008) that could have resulted in a reduction of the cell division rate. However, both untreated and colchicine-treated *ex vitro* plants were viable and grew equally well under greenhouse conditions, suggesting that, for this hybrid, colchicine could be affecting growth negatively only in the initial stages. This contradicts the observations reported for other genera and species, such as *Mecardonia tenella* (Escandón et al., 2007) *Bacopa monnieri* (Escandón et al., 2006) and *Scoparia montevidiensis* (Escandón et al., 2005), in which the reduction of growth rates in the colchicine-treated plants was always noticeable, even when the plants were in the flowering period. For the *Glandularia* hybrid, there was only a very small delay in flowering, which could be attributed to the larger size of the inflorescences of the polyploid plants. All the subjects recovered from the control treatments were diploid, and some individuals that had been treated with colchicine were also diploid. It is important to highlight that no differences were detected between the diploid

individuals recovered either from the control or colchicine treatments.

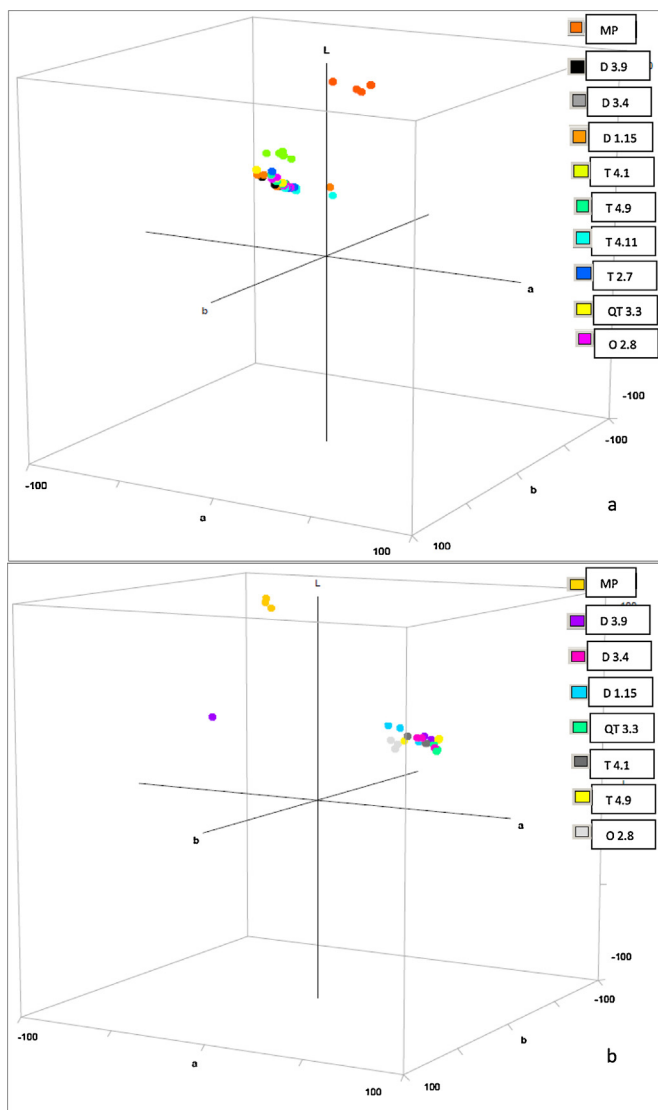
It was observed that, while different doses of colchicine were equally effective, treatments 2 (0.001% colchicine 48 h) and 4 (0.01% colchicine 48 h) appeared to be the most adequate in terms of the number of polyploid individuals recovered. This would suggest that, in terms of treatment efficacy, the exposure time would prevail over the alkaloid concentration in this particular case.

The fact that no differences were observed in stem diameters or leaf area indices would suggest that, at least for *Glandularia* hybrid and, contrary to our previous reports (Escandón et al., 2005, 2006, 2007), these traits cannot be considered good parameters to differentiate diploid from tetraploid individuals. In regard to the leaves, the most noticeable feature distinguishing polyploid from diploid *Glandularia* hybrid plants was that the leaf tissue of the former was thicker and more consistent. All these findings were unexpected since the size, color and thickness of the leaves along with the stem diameter are usually considered strong and direct parameters to identify individuals with different ploidy levels in many other species (Escandón et al., 2007; Eeckhaut et al., 2006; Horn, 2002). In the present study, some organs or cells exhibited the expected response (flowers, stomata and pollen grain), but other organs (leaves and stem) showed no significant differences between diploid and tetraploid plants. This dichotomous response (normal organs/enlarged organs) could be due to the fact that the formation of new synthetic polyploid plants may be accompanied by random changes in genome organization and rearrangements and changes in gene expression (Chen and Zhongfu, 2006; Dhooghe et al., 2011).

With regard to the difference between the color of leaves and flowers of the *ex vitro* plants, both diploid and tetraploid, in relation to the mother plant, it can be speculated that the *in vitro* culture in itself could generate changes in plant pigmentation, in this case resulting in darker colors (both leaf and flower) compared to the original individual.

Using the protocol presented in this paper a *Glandularia* hybrid with larger flowers and inflorescences was obtained. Furthermore, more than 12 months after obtaining the new genotypes, these phenotypic characteristics remained, thus allowing their incorporation into a *Glandularia* breeding program.





**Fig. 7.** The graphics show the distribution of the individuals of the plant populations recovered according to the color of leaves (a) and flowers (b). MP = mother plant in both cases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 5. Conclusions

In the present work, an optimized propagation and polyploidization protocol for an interspecific *Glandularia* hybrid is presented. The nutritional requirements were consistent with previous results obtained working on other *Glandularia* species. The results also suggest that, at least for the species studied, *Glandularia* has very specific PGR demands. The duplication of genetic information was not correlated with the duplication of the traits studied in the hybrid. From the point of view of the ornamental requirements, plants with larger flowers and inflorescences were obtained. Tetraploid plants are a valuable and promising material and were included in a *Glandularia* breeding program.

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