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ORIGINAL ARTICLE

Production of compact petunias through polyploidization

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Abstract Petunia, a commercially important ornamental plant worldwide, has been subjected to breeding programs that have yielded a high number of varieties. One of the key factors in the commercial value of these varieties is plant compactness. Currently, compact petunias are obtained through the application of expensive, harmful and short-lasting chemicals. To avoid the use of these chemicals, transgenic plants that over-express dwarf-inducing genes have been recently proposed as an alternative, but the current legislation regarding transgenic plants restricts their commercialization. In this work, we studied the effect of polyploidization in the plant architecture of *Petunia axillaris*, an Argentine native petunia. We developed a new polyploidization protocol that consisted in culturing petunia leaves in RL medium (MS medium supplemented with 30 g l^{-1} sucrose, 1 mg l^{-1} BA and 0.2 mg l^{-1} IAA) supplemented with 0.2 g l^{-1} colchicine for 15 days. This protocol allowed the regeneration of stable autotetraploid petunias (polyploidization rate: 29.0 ± 8,2%), which were 54% more compact than the diploid ones. Furthermore, they exhibited no variations in agronomical traits compared to the initial genotypes, except for a short delay in blooming. These autotetraploid plants can be used in different breeding programs and the polyploidization method developed can be tested in others cultivars of the genus *Petunia* for the same purpose.

Keywords Colchicine · *Petunia axillaris* · Autotetraploids · Plant architecture · Ornamentals · Organogenesis · Plant compactness, dwarfing

Introduction

The common garden petunia, *Petunia hybrida*, is one the most popular ornamental plants in the world. Only in the USA, the wholesale value of petunia in 2015 was U$133 million (USDA-NASS 2016). Petunia presents abundant flowering from early spring to late autumn. Depending on the variety, the flowers display a large array of colors. This plant is considered the first cultivated bedding plant (Kelly et al. 2007) and its importance has led to the continuous development of new varieties. Breeding began in the 1800s using two South American species (*Petunia axillaris*, with white flowers, and *P. integrifolia*, with purple ones) and it eventually led to the development of over 360 cultivars of petunia available on the market today (Kelly et al. 2007). Nonetheless, the market continuously requires new cultivars. To meet this demand the Institute of Floriculture, which is part of the National Agricultural Technology Institute of Argentina (INTA), conducts a breeding program to obtain new cultivars from native Argentine species. This program has established a native ornamental germplasm bank in which there are five native species of petunias: *P. parviflora, P. infirata, P. interior, P. integrifolia and P. axillaris* (Fernandez et al. 2008).

As with other potted plant species, petunia has become a challenge for the flower-growers who seek to increase the quality of their production with more compact plants

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(Francescangeli and Zagravia 2008). Currently, chemical growth regulators are widely used to modify plant stature (Rademacher 2000). One of the most commonly employed growth regulators is paclobutrazol, a gibberellin synthesis inhibitor (Schnelle et al. 2010; Francescangeli and Zagabria 2008). The use of these growth regulators increases the investment in crop production due the cost of the chemicals and the labor needed to apply them (Bhattacharya et al. 2010). Moreover, the effect of these growth regulators is not permanent, and repeated applications may be necessary to achieve the desired results (Radi 2005). Finally, these chemicals are classified as pesticides and their use is rigorously regulated (Bhattacharya et al. 2010), since they are dangerous to human health (Andersen et al. 2002). Consequently, the search for alternatives to the use of growth regulators is essential.

In the last years, overexpression of different dwarf-inducing genes has been proposed as an alternative to growth regulators to obtain compact petunias. Compact petunias have been obtained through transformation with *Agrobacterium rhizogenes rol* genes (Winefield et al. 1999; Khan et al. 2010) and *A. tumefaciens* harboring different genes: *GA_2ox* from *Nicotiana tabacum* (Gargul et al. 2013) and *AtCBF3* from *Arabidopsis thaliana* (Walworth et al. 2014). The current legislation regarding transgenic plants, and the negative perception of these plants among consumers, constitute important limitations to the commercialization of these transgenic compact petunias. This is particularly true in the European Union, which is the largest producer and consumer of ornamental plants.

Polyploidization has been used, with different results, to produce changes in the floral architecture of bedding plants. Autopolyploid plants have been developed in several species, such as *Caladium* (Cai et al. 2015), *Glandularia* (González-Roca et al. 2015), *Oratia Beauty* (Gallone et al. 2014), *Gerbera* (Gantait et al. 2011) and *Mercadonia tenella* (Escandón et al. 2007). In petunia, autopolyploid plants displaying compact phenotypes have been obtained by both treating seeds with colchicine (Ning et al. 2009) and regenerating plantlets from isolated protoplasts (Meyer et al. 2009; Oh et al. 1995). The treatment of petunia seedlings with colchicine produced an important number of polyploid petunias, but most of the plants obtained were chimeras and the leaves presented serious malformations (Ning et al. 2009). Generally, the use of multicellular organs or tissues for polyploidization results in chimeras or mixed ploidy levels (Broertjes and Keen 1980; Cai et al. 2015), and this has been reported for different bedding plants, such as *Glandularia* (González-Roca et al. 2015), *Hebe* (Gallone et al. 2014) or *Mercadonia tenella* (Escandón et al. 2007). To obtain autopolyploid plants without chimeras, organogenesis from leaf discs grown in culture media supplemented with colchicine is a good alternative, since the shoots regenerate from a few cells. Shoot regeneration from petunia leaves is a well developed technique (Beck and Camper 1991; Reuveni and Evenor 2007), but it has never been used to regenerate autopolyploid petunias.

The objective of this work was the development of a polyploidization protocol to obtain petunias with a more compact architecture, while maintaining the other traits of the initial cultivars. We used an Argentine native species, *P. axillaris*, because its outward appearance allows a better assessment of changes in compactness.

Materials and methods

Plant material

The leaves used for polyploidization derived from in vitro plantlets of *P. axillaris* ($2n = 2\times = 14$), obtained from the native ornamental germplasm bank established by the Institute of Floriculture (INTA). Every 4 weeks, the plantlets were subcultured, in individual test tubes, containing 10 ml of MS (Murashige and Skoog 1962) culture medium supplemented with 30 g l^{-1} sucrose, solidified with 8 g l^{-1} of bacteriological agar and pH adjusted to 5.74 before autoclaving. The pH and the gelling agent were the same in all culture media used in this study. The cultures were grown in an incubator model I-291PF (Ingelab) at 25 ± 2 °C under 16:8 h photoperiod using white fluorescent tubes with a light intensity level of 40 µmol photon m^{-2} s^{-1}. The incubation conditions were the same throughout all the assays.

Regeneration and multiplication of autopolyploid petunias

To obtain autopolyploid petunias, 50 leaves of *P. axillaris* were cultured individually in tubes, on 10 ml of RL (regeneration leaves) culture media, supplemented with 0.2 g l^{-1} colchicine (dissolved in water and filter sterilized) for 15 or 30 days, with 25 leaves for each treatment. The composition of the RL medium consisted of MS basal medium supplemented with, with 30 g l^{-1} sucrose, 1 mg l^{-1} BA (6-benzyladenine) and 0.2 mg l^{-1} IAA (Indole-3-acetic acid). The control consisted of 25 leaves cultured in RL media without colchicine. After colchicine treatment, the leaves were transferred to test tubes with 10 ml of RL media without colchicine for 4 weeks. Finally, data concerning contamination, survival rate and shoot regeneration was recorded.

The regenerated shoots were cultured individually, in test tubes, on 10 ml of MS medium (Murashige and Skoog 1962) deprived of growth regulators and supplemented with 30 g l^{-1} sucrose. Once rooted, the regenerated petunias were multiplied by culturing individual nodal segments in the same culture media every 4 weeks. Each regenerated

shoot was considered an independent regeneration event. As control, 25 shoots regenerated from leaves cultured in RL medium without colchicine were rooted and multiplied. The number of nodal segments obtained from each plantlet was registered.

Flow cytometry analysis

The ploidy level of the different regeneration events from leaves cultured with colchicine was determined by estimating the relative DNA content using flow cytometry (Ploidy Analyser PA-I; Partec GmbH, Münster, Germany). In addition, the ploidy level of the 25 control shoots regenerated from leaves cultured on RL without colchicine was determined. To release nuclei, young in vitro leaves, 0.5 cm^2, were chopped with a razor blade for 30–60 s in a Petri dish containing 0.4 ml of nuclei isolation buffer (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, extraction buffer). The homogenate was filtered through a 50 µm nylon mesh (Partec 50-lm CellTrics disposable filter), and subsequently the nuclei were stained with fluorescent dye (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, staining buffer, about 1.6 ml). Finally, the samples were analyzed after 30 s of incubation. As an external standard, leaves from the *P. axillaris* (2n = 2× = 14) mother plants were used. This analysis was repeated after acclimatization of the diploid and autotetraploid petunias.

Study of stomatal size

The stomatal sizes of six events of regeneration determined as diploid (2n = 2× = 14) and six events determined as autotetraploid (2n = 4× = 28) were measured. The stomatal sizes of three petunias (2n = 2× = 14) regenerated from leaves cultured in RL medium without colchicine and one "normal" plant of *P. axillaris* (2n = 2× = 14) were measured as controls. The length of the stomata was measured in epidermal strips prepared according to Gudesblat et al. (2006). In vitro leaves were briefly rinsed (1′ approximately) on a paper towel and stuck through their abaxial surface to one of the two adhesive sides of a tape (the other side of the tape had previously been stuck to a cover slip). Using a razor blade, all leaf tissues except the abaxial epidermis were rapidly removed. The epidermis layers were moistened with a few drops of water, and the cover slip was subsequently placed on a microscope slide. Thirty randomly selected stomata from each plantlet were measured in an Olympus BX41 microscope (x400). The photographs were taken with a Camera Infinity 1 and the stomata were measured using Infinity Analyze software package (version 6.3.0; 2006–2013 Lumenera Corporation, Ottawa, Canada).

Acclimatization and characterization of autopolyploid petunias

Acclimatization of the plantlets was carried out, during the spring of 2016, in the greenhouses of the Institute for Mediterranean and Subtropical Horticulture "La Mayora" (IHSM-UMA-CSIC), Algarrobo-Costa (Malaga - Spain). A total of 30 diploid (2n = 2× = 14) and 30 autotetraploid petunias (2n = 4× = 28) were thoroughly washed in tap water and transplanted to 4 × 4 cm polyethylene alveolus trays containing a mixture of autoclaved peat: perlite (1:1). Potted plantlets were maintained for 1 month in a polyethylene tunnel with 80% relative humidity. The temperature inside the tunnel ranged from 19 to 30 °C, and the mean temperature was 25 °C. Afterwards, the rate of acclimatization was registered. The acclimatized plants were transferred to 12 cm diameter pots containing the same substrate and maintained at 50–60% relative humidity for another 2 months. During this period, the following traits were characterized in ten diploid and ten autotetraploid petunias: plant height, number of nodes, number of leaves, leaf surface area in the larger leaves, days to flowering after acclimatization, number of flowers and flower diameter. The first data were collected 2 months after acclimatization, in coincidence with the first flowering of an autotetraploid petunia. The data were collected each week for 4 weeks. Finally, plant compactness was calculated as the dry weight of the petunias per unit of plant height (Mata and Botto 2009; van Iersel and Nemale 2004). The dry weight was determined by drying the plants at 65 °C for 4 days.

Statistical analysis

All data were analyzed using SPSS software package (version 19.0; SPPS INC., Chicago, IL, USA). The rates of contamination, regeneration and polyploidization obtained in the different polyploidization assays were analyzed by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution. Pairwise comparisons among groups were performed by Fisher's least significant difference (LSD) test. Both the number of shoots regenerated from each leaf and the number of nodal segments obtained from the plantlets were analyzed by one-way ANOVA, using a HSD-Tukey test in the post-hoc analysis for comparisons among groups. The same analysis was used to analyze the stomatal sizes of the different events of regeneration

Table 1 Contamination, regeneration and polyploidization rates and number of plantlets regenerated in the different polyploidization assays from leaves of *P. axillaris* cultured in RL medium supplemented with 0.2 g l^{-1} of colchicine for 15 or 30 days

Treatment duration	Contamination rate (%)[1]	Regeneration rate (%)[1]	Nº plantlets regenerated[2]	Polyploidization rate[1]	No. regeneration events autotetraploids/total regeneration events
Control (Without colchicine)	4 ± 3.9^{b}	100 ± 0^{a}	$33{,}2 \pm 8.2^{a}$	$0 \pm 0\%^{b}$	0/797
15 days	32 ± 9.3^{a}	47 ± 12.1^{b}	$3{,}9 \pm 0.8^{b}$	$29 \pm 8.2\%^{a}$	9/31
30 days	40 ± 9.8^{a}	7 ± 6.4^{c}	1[3]	–[4]	1/1

[1]Different letters indicate groups that were significantly different by LSD at a = 0.05.

[2]Different letters indicate significant differences by one-way ANOVA, using a HSD-Turkey test in the post-hoc analysis for comparisons among groups

[3]Only one regeneration event was registered in the 30-day trial (no statistical analysis was possible)

[4]Polyploidization rate could not be estimated because only one regeneration event was registered

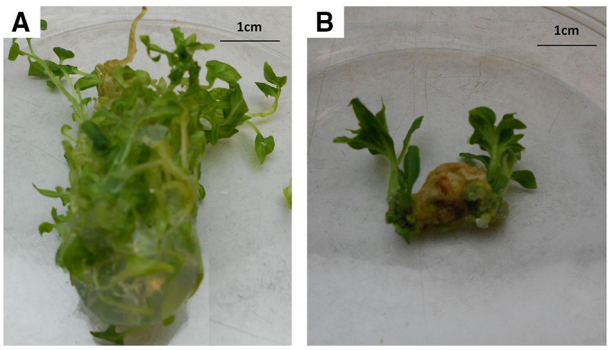


Fig. 1 **a** *P. axillaris* leaf after 30 days of culture in RL media (Control). **b** *P. axillaris* leaf after 30 days of culture in RL media supplemented with colchicine

and for the characterization of the autotetraploid petunias obtained in this work.

Results

Production of autotetraploid petunias

Contamination rates increased substantially when the culture media was supplemented with colchicine. Contamination rates in the control were less than 5%, compared to the culture media supplemented with colchicine, where values reached above 30%, irrespective of treatment length (Table 1). Colchicine also reduced the regeneration capacity of petunia leaves, both in the percentage of leaves with regeneration as in the number of regeneration events produced in each leaf (Table 1; Fig. 1). This effect was proportional to the duration of the treatment with colchicine. All the control leaves exhibited shoot regeneration (100%), with an average of 33.2 ± 8.2 regeneration events per leaf. Regeneration was successful in $47.0 \pm 12.1\%$ of the leaves cultured for 15 days on RL media supplemented with colchicine, but only 3.9 ± 0.8 regeneration events per leaf were recorded. Finally, in the leaves cultured for 30 days on RL media supplemented with colchicine, there was only one regeneration event in one leaf ($7.0 \pm 6.4\%$).

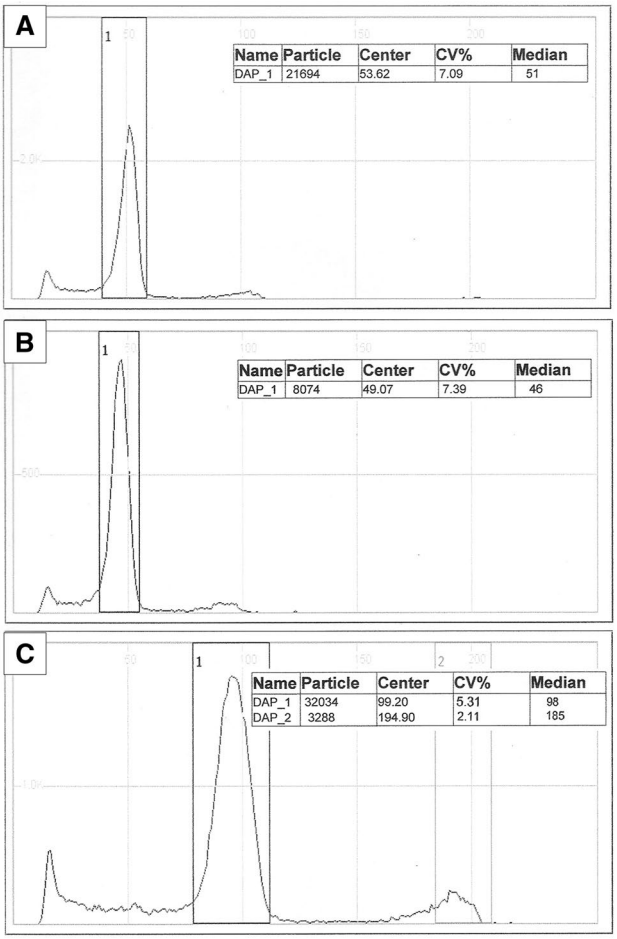


Fig. 2 Flow cytometry histograms for three *P. axillaris* plantlets: **a** Control diploid plant of *P. axillaris* (2n = 2× = 14), **b** diploid petunia (2n = 2× = 14) regenerated from leaf cultured in RL media without colchicine, **c** autotetraploid petunia (2n = 4× = 28) regenerated from leaf cultured for 15 days in RL media supplemented with colchicine

The results of flow cytometry assays are shown in Fig. 2 and Table 1. Leaves of in vitro plants of *P. axillaris* (2n = 2× = 14) were used as an external standard in all ploidy level determinations. In the flow cytometry histogram, a value of 50 was established as the one corresponding to the peak of the G1 somatic nuclei (Fig. 2a). The leaves used to determine ploidy level were those whose growth had concluded, thus ensuring few G2 somatic nuclei. Consequently, the peak corresponding to these G2 somatic nuclei, which should appear at a value of 100, was hardly visible in the histogram. Ploidy level analysis of 25 shoots, randomly selected among the 797 regeneration events produced in leaves cultured in RL without colchicine, revealed that they were all diploid (2n = 2× = 14) (0% the polyploidization rate) (Table 1). The flow cytometry histogram corresponding to these diploid petunias (Fig. 2b) was similar to the flow cytometry histogram of the diploid control (Fig. 2a). There were 31 (29.0 ± 8.2%) regeneration events in the leaves cultured in RL supplemented with colchicine for 15 days. The flow cytometry histogram of nine of these events indicated that the shoots were autotetraploid (2n = 4× = 28). The flow cytometry histogram of the autotetraploid shoots showed a main peak situated at a value of 100, representing G1 tetraploid somatic nuclei (2n = 4× = 28), and a secondary peak located at 200, representing the few G2 tetraploid somatic nuclei (4n = 8× = 56) (Fig. 2c). Finally, the only shoot that regenerated from the leaves cultured in RL supplemented with colchicine for 30 days was tetraploid (Table 1).

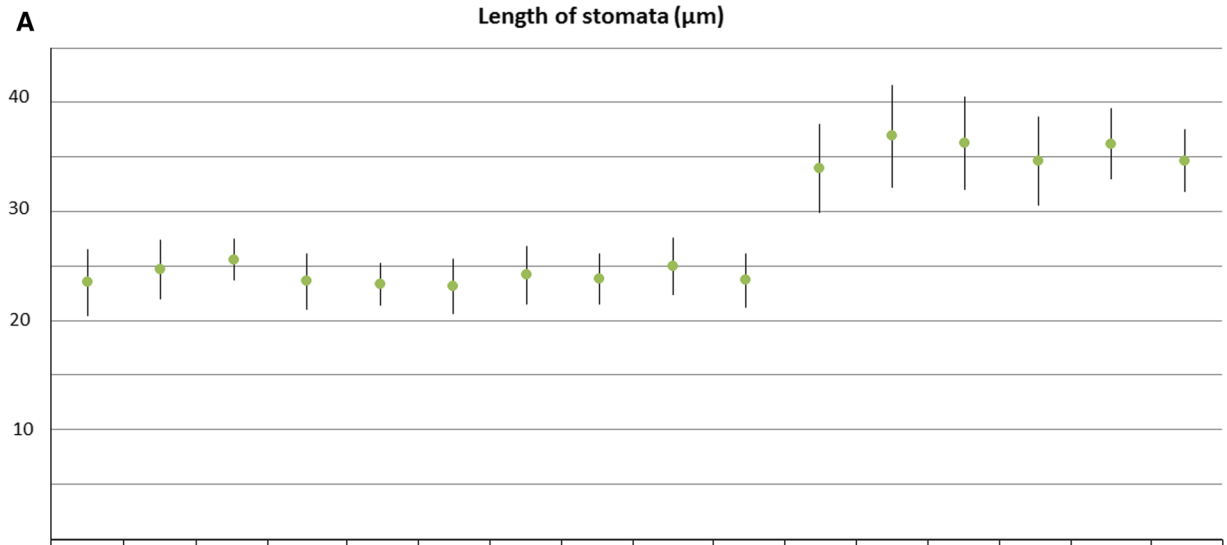
The multiplication rate of diploid petunias (7.1 ± 1.8) was much higher than in autotetraploid ones (2.6 ± 0.7), because the compact architecture of the autotetraploid petunias hinders the separation of nodal segments. The autotetraploid petunias had more compact aerial parts than the diploid plants (Fig. 3). They had a similar number of leaves, but the internodes were shorter. The roots of diploid and autotetraploid petunias were very similar. After acclimatization of diploid and autotetraploid petunias, a more exhaustive characterization will be performed to confirm a more compact phenotype in mature autotetraploid petunias.

Stomatal sizes of diploid and autotetraploid petunias are shown in Fig. 4. The stomata of the *P. axillaris* diploid control plants (2n = 2× = 14) had a length of 23.51 ± 3.04 µm (Fig. 4B). All diploid petunias, regenerated from leaves cultured on RL media with and without colchicine for 15 days, exhibited stomata with similar lengths and without statistically significant differences with respect to the stomata of the control plants (Fig. 4a, c). The autotetraploid petunias (2n = 4× = 28) had significantly bigger stomata than the diploid petunias (Fig. 4a, d), with lengths ranging from 33.96 ± 4.04 to 36.93 ± 4.04 µm and non-significant differences between the different autotetraploid regenerants.

Fig. 3 Diploid (*left*) and autotetraploid (*right*) plants of *P. axillaris* grown in vitro in MS media (Murashige and Skoog 1962) supplemented with 30 g l^{-1} of sucrose and ready for acclimatization

Characterization of autotetraploid petunias

All petunias, both diploid and autotetraploid, were successfully acclimatized (100%). Autotetraploid petunias were characterized 8 weeks after acclimatization. The first feature analyzed was plant height in relation to the number of internodes (Fig. 5). The height of both diploid and autotetraploid petunias increased during the 8 and 9th weeks, but there were always significant differences between the two ploidy levels (Fig. 5). Plant height stabilized between the 10 and 11th weeks, but maintained the significant differences between diploid plants (83.3 ± 8.8 cm) and autotetraploid plants (47.3 ± 7.8 cm). However, the number of internodes remained constant during the 4 weeks studied without significant differences between diploid and autotetraploid plants (Fig. 5). These results suggest that, in the 4-week period analyzed, the increase in height was due to elongation of the internodes and not to the formation of new internodes.



Petunia axillaris (2x)	REOC 1	REOC 2	REOC 3	RE1 (2X)	RE2 (2X)	RE3 (2X)	RE4 (2X)	RE5 (2X)	RE6 (2X)	RE1 (4X)	RE2 (4X)	RE3 (4X)	RE4 (4X)	RE5 (4X)	RE6 (4X)
23.51 ±3.04[a]	24.68 ±2.71[a]	25.60 ±1.84[a]	23.58 ±2.56[a]	23.31 ±1.94[a]	23.14 ±2.53[a]	24.20 ±2.65[a]	23.79 ±2.31[a]	25.00 ±2.62[a]	23.70 ±2.44[a]	33.96 ±4.04[b]	36.93 ±4.68[b]	36.30 ±4.27[b]	34.61 ±4.05[b]	36.21 ±3.22[b]	34.63 ±2.85[b]

*Different letters (a, b) indicating statistically significant differences by one-way ANOVA, using an HSD-Turkey test in the post-hoc analysis for comparisons among groups.

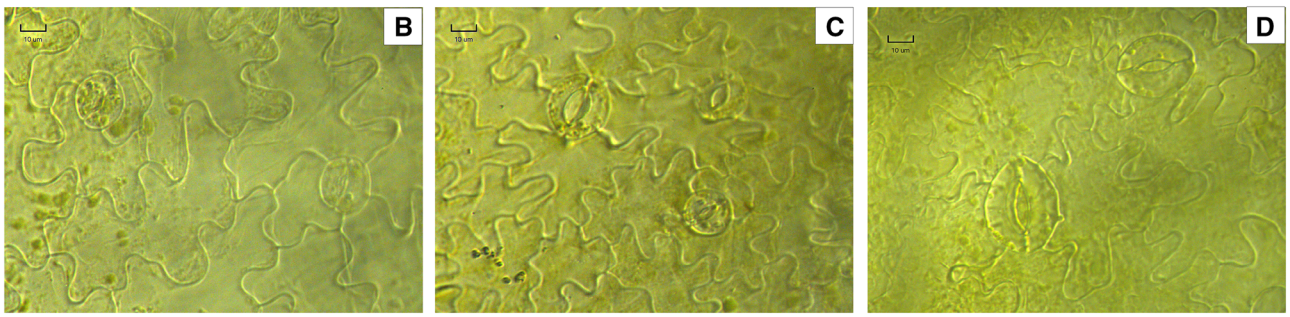
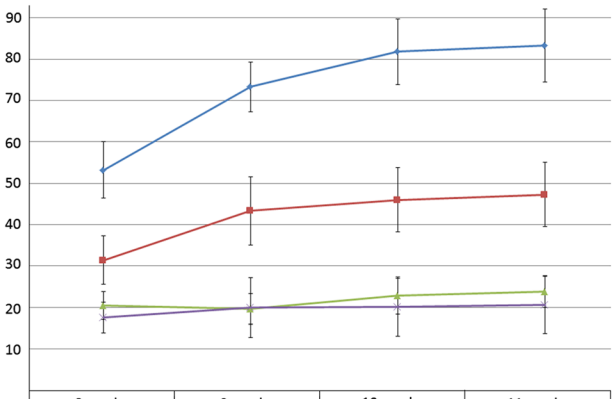


Fig. 4 Study of the size of the stomata of diploid and autotetraploid *P. axillaris*. **a** Length of stomata (µm) of the different petunias analyzed: control diploid *P. axillaris* (2n = 2× = 14), diploid REOC (regeneration events in RL media without colchicine) (2n = 2× = 14), 2× RE (diploid regeneration events in RL media supplemented with colchicine) (2n = 2× = 14) and 4× RE (autotetraploid regeneration events in RL media supplemented with colchicine) (2n = 4× = 28). **b** Stomata from control diploid *P. axillaris* (2n = 2× = 14) (×400). **c** Stomata from diploid *P. axillaris* (2n = 2× = 14) (×400). **d** Stomata from autotetraploid *P. axillaris* (2n = 4× = 28) (×400)

The number of leaves per plant increased during the growth period studied, with values that ranged from less than 20 at the beginning to around 30 at the end of the period. There were no statistically significant differences, both in the number and size of the leaves, between diploid and autotetraploid plants (Fig. 6). Likewise, no significant differences were detected between the leaf surface area in diploid and autotetraploid plants (Fig. 6). The reduction of the leaf surface area observed from the 9 to the 11th week was due to the formation of new leaves that were smaller than the basal leaves of the petunias. These results suggest that polyploidization has no effect on the number or size of leaves of petunia.

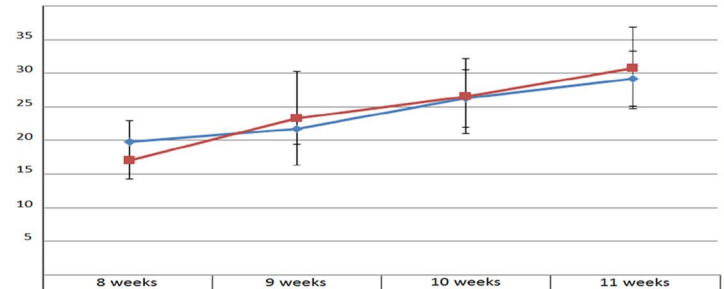
Flowering time was affected by polyploidization. Diploid petunias flowered 39.7 ± 3.86 days after acclimatization, while blooming was delayed in autotetraploid petunias (65.90 ± 5.95 days after acclimatization). As a result, diploid petunias had 4.1 ± 1.9 flowers per plant when analyzed for the first time, while the autotetraploid petunias had 0.7 ± 0.7 flowers (Fig. 7). However, when the study ended at week 11, the number of flowers per plant in autotetraploid petunias was above four and there were no significant differences with respect to the diploid ones. These results indicate that while polyploidization delayed flowering, it did not affect the final number of flowers per plant. Similarly, the diameter of the flowers was not affected by polyploidization (Fig. 7). Flower diameters ranged between 5.5 and 6 cm, without significant differences between diploid and autotetraploid petunias.



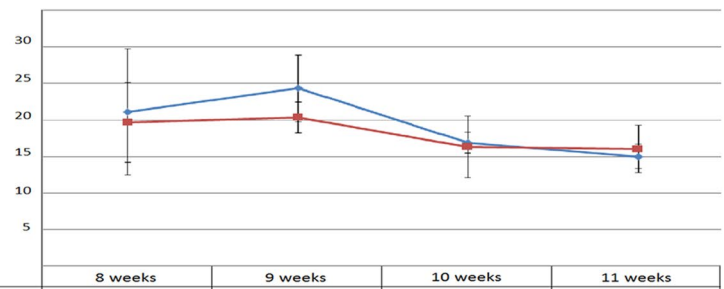
	8 weeks	9 weeks	10 weeks	11 weeks
Diploid plants height (2n=2x=14) (cm)	53.2 ± 6.8 [a]	73.3 ± 6.0 [a]	81.8 ± 7.9 [a]	83.3 ± 8.8 [a]
Autotetraploid plants height (2n=4x=28) (cm)	31.4 ± 5.9 [b]	43.3 ± 8.2 [b]	46.0 ± 7.8 [b]	47.3 ± 7.8 [b]
Diploid plants number of internodes (2n=2x=14)	20.4 ± 3.4 [a]	19.6 ± 3.7 [a]	22.9 ± 4.5 [a]	23.8 ± 3.8 [a]
Autotetraploid plants number of internodes (2n=4x=28)	17.5 ± 3.7 [a]	20.0 ± 7.2 [a]	20.1 ± 7.0 [a]	20.6 ± 6.9 [a]

*Different letters (a, b) indicating statistically significant differences by one-way ANOVA between diploid and autotetraploid plants in the same week

Fig. 5 Study of the height and number of internodes in diploid and autotetraploid plants of *P. axillaris* between the 8 and 11th week after acclimatization of plants. **a** Diploid plant of *P. axillaris* 11 weeks after acclimatization. **b** autotetraploid plant of *P. axillaris* 11 weeks after acclimatization



	8 weeks	9 weeks	10 weeks	11 weeks
Number of leaves diploid plants (2n=2x=14)	19.8 ± 3.2 [a]	21.7 ± 2.3 [a]	26.3 ± 4.3 [a]	29.2 ± 4.1 [a]
Number of leaves autotetraploid plants (2n=4x=28)	17.0 ± 2.8 [a]	23.3 ± 7.0 [a]	26.6 ± 5.6 [a]	30.8 ± 6.1 [a]



	8 weeks	9 weeks	10 weeks	11 weeks
Leaf surface (cm²) diploid plants (2n=2x=14)	21.08 ± 8.64 [a]	24.32 ± 4.59 [a]	16.88 ± 1.42 [a]	15.00 ± 1.67 [a]
Leaf surface (cm²) autotetraploid plants (2n=4x=28)	19.68 ± 5.45 [a]	20.32 ± 2.13 [a]	16.32 ± 4.23 [a]	16.04 ± 3.27 [a]

*Values showing the same letter indicating that there were no statistical significant differences by one-way ANOVA between diploid and autotetraploid plants in the same week.

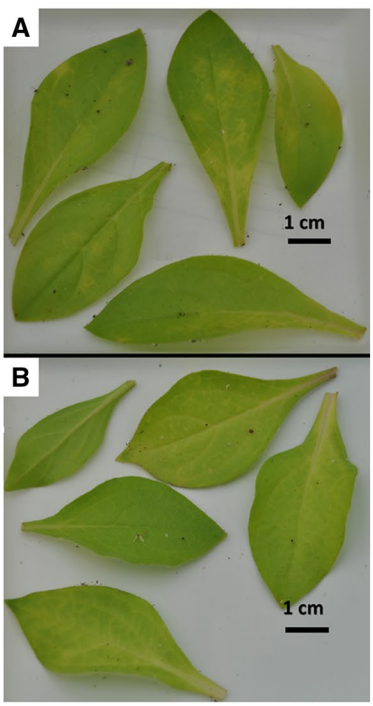
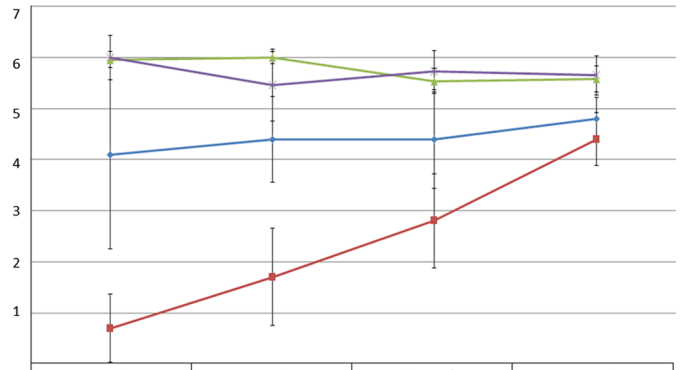


Fig. 6 Study of the number of leaves and leaf surface in diploid and autotetraploid plants of *P. axillaris* between the 8 and 11th week after acclimatization of plants. **a** Leaves of diploid plant of *P. axillaris* 11 weeks after acclimatization. **b** Leaves of autotetraploid plant of *P. axillaris* 11 weeks after acclimatization



	8 weeks	9 weeks	10 weeks	11 weeks
Number of flowers diploid plants (2n=2x=14)	4.1 ± 1.9 [a]	4.4 ± 0.9 [a]	4.4 ± 1.0 [a]	4.8 ± 0.4 [a]
Number of flowers autotetraploid plants (2n=4x=28)	0.7 ± 0.7 [b]	1.7 ± 1.0 [b]	2.8 ± 0.9 [b]	4.4 ± 0.5 [a]
Flower diameter (cm) diploid plants (2n=2x=14)	5.96 ± 0.16 [a]	6.00 ± 0.12 [a]	5.54 ± 0.25 [a]	5.58 ± 0.26 [a]
Flower diameter (cm) autotetraploid plants (2n=4x=28)	6.00 ± 0.44 [a]	5.46 ± 0.71 [b]	5.73 ± 0.41 [a]	5.65 ± 0.38 [a]

*Different letters (a, b) indicating statistically significant differences by one-way ANOVA between diploid and autotetraploid plants in the same week.

Fig. 7 (**a**) Study of the number and diameter of flowers in diploid and autotetraploid plants of *P. axillaris* between the 8 and 11th week after acclimatization of plants. (**b**) Flower of tetraploid *P. axillaris*

Finally, plant compactness of diploid and autotetraploid petunias was calculated. The value was 0.035 ± 0.005 for diploid petunias and 0.054 ± 0.010 for autotetraploid petunias. These results confirmed that polyploidization increased the compactness of *P. axillaris* (54%).

Discussion

The major drawback of polyploidization using colchicine is the common generation of undesirable mixoploid plants. Furthermore, these mixoploid plants are unstable, since frequently the competition between the original cells and the polyploid ones finally eliminates the polyploid cells. The use of multicellular tissues or organs as initial explants increases the mixoploid rates (Broertjes and Keen 1980; Cai et al. 2015). For example, Ning et al. (2009) used petunia seedlings as initial explants and obtained a mixoploid rate of 80%, but only 20% of the plants were tetraploid. Morphological changes can also derive from natural endoreduplication, a common phenomenon in in vitro cultures of several species, such as the propagation of orchids of the genus *Phalaenopsis* (Lee et al. 2016) or the callus culture of *Asparagus* sp. (Regalado et al. 2015) and *Gentiana decumbens* L.f. (Tomiczak et al. 2015, 2016). For instance, Meyer et al. (2009) regenerated diploid, tetraploid and hexaploid plants from the culture of isolated diploid protoplasts and detected morphological changes in all of them, including the diploid plants, probably due to somaclonal variation and protoplast fusion.

To avoid the problems cited above, we regenerated shoots from leaf explants cultured in media supplemented with colchicine. In Fig. 1, we can observe that most of the leaf cells (derived from 15-day colchicine treatment) died due to colchicine toxicity, and only a few surviving cells regenerated into shoots. As a result, all the regenerated plantlets with different ploidy levels were tetraploid (Fig. 2), thus circumventing the formation of mixoploid plants.

The increase in the polyploidization rate is another advantage of this method. In Petunia, Ning et al. (2009) obtained a polyploidization rate of 20% using seedlings cultured in colchicine and Meyer et al. (2009) obtained a polyploidization rate of 7.5% through the regeneration of plantlets from isolated protoplasts. We obtained a polyploidization rate of 29.0 ± 8.2% when the leaves were cultured in the media supplemented with colchicine for 15 days and a rate of 100% when this period was extended to 30 days. However, the 30-day culture in media supplemented with colchicine resulted in a high rate of cell death and, consequently, a low regeneration rate (7.0 ± 6.4%). Only one autotetraploid petunia regenerated from the only leaf with surviving cells after 30 days of treatment. In the 15-day treatment, nine autotetraploid plants were obtained from a total of 31 regeneration events, making this the preferred treatment. Finally, no autotetraploid regeneration event was detected among the 797 regenerants recorded in

the control leaves. Contrary to what was observed in the regeneration of petunias from protoplasts (Oh et al. 1995; Meyer et al. 2009), these results suggest that the regeneration of shoots from petunia leaves cultured in RL media does not induce endoreduplication. Therefore, colchicine is recommended to induce polyploidization.

We used flow cytometry to determine the ploidy level of the different regeneration events This technique is faster and easier than traditional cytogenetic techniques employed to analyze the ploidy level of plants (Ochatt et al. 2011), and has already been used in previous publications within the genus *Petunia* (Chen et al. 2014; Hoshino et al. 2016). Although stomatal size is another technique that has been used to establish ploidy levels (Detrez et al. 1989; Doleẑel et al. 1994; van Duren et al. 1996), it is usually not reliable enough (Ochatt 2006, 2008). In our case, the stomatal size of the different regenerants showed important differences between the autotetraploid and the diploid petunias, with larger stomata observed in autotetraploid petunias Nevertheless, we recommend the use of flow cytometry as we consider this technique more reliable.

The autotetraploid petunias regenerated in vitro presented a much more compact architecture than the diploid ones. Usually, the differences detected between in vitro grown plants are preserved in the phenotype of the acclimated adult plants. We can find examples of this in different works within the genus *Asparagus* (Carmona-Martin et al. 2015; Regalado et al. 2015). Nevertheless, we decided to micropropagate and transplant autotetraploid and diploid plantlets to analyze the effect of polyploidization in the structure of the adult petunias.

The mature autotetraploid petunias were subjected to a thorough characterization that included the following features: plant height, number of internodes, number of leaves, leaf surface area, days to flowering, number of flowers and flower diameter. The main effect of polyploidization was a significant decrease in the height of the autopolyploid petunias due to the shortening of the internodes, although their number remained unchanged. Polyploidization produces similar effects in some ornamentals such as *Mercadonia tenella* (Escandón et al. 2007) or *Lobularia maritima* (Huang et al. 2015), and opposite ones in other species such as *Lagerstroemia indica* (Ye et al. 2010) or *Gerbera jamesonii* (Gantait et al. 2011). Previous polyploidization research works in the genus *Petunia* showed similar results to ours regarding height decrease (Ning et al. 2009). The polyploidization method used in this work did not cause changes in the number of leaves or in their form and surface area (Fig. 6). In contrast, these malformations were present in the autopolyploid petunias produced by other polyploidization methods, such as the regeneration of plantlets from protoplasts (Meyer et al. 2009; Oh et al. 1995) or seeds treated with colchicine (Ning et al. 2009). These results indicate that our polyploidization method produced autotetraploid petunias that were more similar to the initial cultivar than the autotetraploid petunias produced with other procedures.

Polyploidization causes an increase in flower diameter in most ornamental species, such as *Gerbera jamesonii* (Gantait et al. 2011), *Glandularia* sp. (González-Roca et al. 2015), *Crocosmia aurea* (Hannweg et al. 2013), *Lagerstroemia indica* (Ye et al. 2010), *Mercadonia tenellas* (Escandón et al. 2007), *Lobularia marítima* (Huang et al. 2015). However, the autotetraploid petunias obtained in this work had flowers with the same diameter than the diploid ones (Fig. 7), and these results are in agreement with previous studies in petunia (Ning et al. 2009). Nevertheless, polyploidization induced a delay of 30 days in flowering in the autotetraploid petunias. This flowering lag has also been described in autopolyploid plants of other species, such as *Glandularia* sp. (González-Roca et al. 2015) or *Gerbera jamesonii* (Gantait et al. 2011). This delay did not affect to final number of flowers per plant, since 11 weeks after acclimatization the number of flowers in the autotetraploid petunias was the same as in the diploid ones (Fig. 7). Nonetheless, in other species the polyploidization can produce a decrease in the number of flowers, as is the case of *Crosocomia aurea* (Hannweg et al. 2013).

Finally, the level of plant compactness recorded for diploid and autotetraploid petunias was analyzed. Usually, plant height is considered a synonym of plant compactness. However, in some studies, smaller plants are more compact (Mata and Botto 2009), but in others this correspondence is not always accurate (van Iersel and Nemali 2004). Therefore, although the differences between the plant height of the autotetraploid and diploid petunias were statistically significant, further analysis of plant compactness was necessary (Fig. 5). Plant compactness was calculated as the dry weight of the petunias per plant height unit. The results obtained confirmed that the autotetraploid petunias were not only shorter but also more compact than the diploid petunias (54%).

In conclusion, the regeneration of shoots from leaves cultured in RL media supplemented with 0.2 g l^{-1} colchicine is a good method to achieve polyploidization of petunia, since it produces a high number of autotetraploid petunias without mixoploid plants. Furthermore, the autotetraploid petunias obtained with this method were more compact than the diploid ones, retaining at the same time most of the traits of the original cultivar, such as the number, color and size of flowers or the number and surface area of leaves. In addition, the autotetraploid plants of *P. axillaris* obtained in this work can be used in different breeding programs and the polyploidization method developed can be tested in others cultivars of the genus *Petunia* to breed more compact petunias. The modified architecture

of these autotetraploid petunias make them an appealing alternative both to the use of growth regulators, which are hazardous to human health, and to the development of transgenic petunias, whose commercialization is highly complicated.

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