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Original Paper

A new variety of *Solanum sisymbriifolium* obtained by *in vitro* polyploidization

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Abstract

Solanum sisymbriifolium is a shrub native to South America and considered a weed of several important crops. Despite this, this species has proven nematicidal capacity and medicinal properties, due to its ability to produce alkaloids. Thus, the objective of this work was to adjust a reliable protocol to obtain *in vitro* polyploid plants of *S. sisymbriifolium* to generate germplasm that serves as the basis for a breeding program of this species aimed to improve the sanitary conditions of horticultural crops with less impact on the environment.

Key words: colchicine, micropropagation, tetraploid, tissue culture.

Resumo

Solanum sisymbriifolium é um arbusto nativo da América do Sul, sendo considerado uma erva daninha para diversas culturas agrícolas. Apesar disto, esta espécie apresenta capacidade nematicida e propriedades medicinais comprovadas devido à produção de alcalóides, com relevante potencial de uso. O objetivo deste trabalho foi obter autoploides sintéticos através da micropropagação e poliploidização *in vitro* de *S. sisymbriifolium*, como base para programa de melhoramento, a fim de aprimorar manejo fitossanitário com menor impacto ambiental.

Palavras-chave: colchicina, micropropagação, tetraplóide, cultura de tecidos.

Introduction

Solanum sisymbriifolium Lam. (n=12, Ratera 1940), also named in Argentina "tutiá", "joâ", "juã", "jurubeba", "revienta caballos", "espina colorada", "tomatillo", "tomate del campo", "fruta de víbora", "agarrate catalina" (Di Ciaccio *et al.* 2021; Sistema Nacional de Vigilancia y Monitoreo de Plagas 2022), is a shrubby plant that belongs to the *Solanaceae* family. It is native to South America (Zuloaga & Morrone 1999), but worldwide distributed. It is a ruderal species that grows along roadsides and in waste places, landfills and ploughed fields. It is commonly pollinated by insects and naturally propagated by seeds, although vegetative propagation by rhizomes is also possible. Due to its vast dispersion and rapid growth, it is considered an invasive weed (Global Invasive Species Database 2022). Despite this classification, *S. sisymbriifolium* is a useful species due to its ability to produce alkaloids. In folk medicine, its roots and/or the entire plant are mostly used as a diuretic, analgesic, contraceptive, antisyphilitic and hepatoprotective (Apu *et al*. 2013). It also has other pharmacological properties related to its empirical uses, such as molluscicidal activity (Bagalwa *et al*. 2010), antimicrobial and antioxidant effects

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(Pasdaran *et al*. 2017), and antihypertensive and anticonvulsant action (Chauhan *et al*. 2011).

Previous reports have shown that *S. sisymbriifolium* can be micropropagated through different techniques of tissue culture (Gleddiei et al. 1985; Deb *et al*. 2019) and protoplast and callus culture (Hag-Hyun & Un-Dong 2005), and that interspecific hybrids (such as *S. sisymbriifolium* × *S. lycopersicum* Lam.) can be obtained through culture of immature embryos (Piosik *et al*. 2019). This perspective would facilitate *in vitro* production of secondary metabolites under controlled conditions and may lead to the application of other biotechnological tools to propagate and/or develop the germplasm. This would allow genetic improvement of the species, generating new genotypes by spontaneous *in vitro* variability or by induction of punctual mutations or polyploidy. Polyploidy, defined as the coexistence of three or more complete sets of chromosomes in the cells of an organism, has played a major role both in the evolutionary history of vascular plants and in the domestication of several crops. In this context, *in vitro* polyploidization has been shown to be an important tool for the development of germplasm of medicinal and aromatic plant species to achieve genotypes with a higher content of secondary metabolites (Iannicelli *et al*. 2020).

On the other hand, modern horticulture requires the development of sustainable alternatives to replace the use of phytosanitary products to control plant parasitic nematodes. In this context, it has been shown that *S. sisymbriifolium* would be a very interesting tool for nematode control (Perpétuo *et al*. 2021). Used as a rootstock, it has a toxic effect on nematodes in several Solanaceae species, such as tomato (Mitidieri *et al*. 2015), eggplant (Timmermans *et al*. 2009; Timmermans 2005), and potato (Dandurand *et al*. 2019). Also, this species has been usefully applied as a biopesticide in bananas (Pestana *et al*. 2014). Recently, Deb *et al.* (2019) regenerated *S. sisymbriifolium* from callus to obtain a large number of rootstocks for tomato grafting.

The importance of *S. sisymbriifolium* as a trap for nematodes and its potential applications as a natural biopesticide and/or as input in the pharmaceutical industry make it necessary to develop its germplasm to obtain new genotypes with greater capacity for the synthesis of active principles.

Based on the references mentioned above on *S. sisymbriifolium in vitro* culture and on the fact that there are no available protocols for this species, the goal of this study was to adjust a reliable protocol to obtain *in vitro* polyploid plants of *S. sisymbriifolium* to generate germplasm that serves as the basis for a breeding program.

Materials and Methods

Plant material

The plant material (young plants from seedlings) was collected at the Instituto Nacional de Tecnología Agropecuaria located in Ituzaingó (34°37'25.8"S 58°40'17.9"W), Buenos Aires, Argentina, with the corresponding permission from the Directorate of Flora and Fauna of the Government of the Province of Buenos Aires (Decree 2020-1702-GDEBA-DSTAPM82529MDGP). The collected material was placed and maintained under greenhouse conditions.

Disinfection process

The plant best adapted to standard greenhouse conditions (natural photoperiod and no control of the temperature) was selected as a source of explants.

The mother plant was sprayed with antifungals (CAPTAN®, 4 ml/l) every two days during the week before *in vitro* introduction.

Nodal segments from non-lignified sectors and young but developed leaves were used as explants. A disinfection protocol was designed for the plant material. First, the explants were kept overnight under running water, then submerged in chloroxylenol solution (4.8%) for 10 min and rubbed with a soft brush. After that, the explants were submerged in 70% ethanol for 1 min and then in 20% sodium hypochlorite plus two drops of Tween 100 for 20 min with sonication. Finally, they were rinsed five times in sterile distilled water in laminar flow. Contamination of the explants was considered in order to evaluate the designed protocol.

In vitro culture

After 30 days of culture, the multiplication stage was initiated with the explants that survived the disinfection.

For all the experiments under *in vitro* conditions, the basal medium was MS culture medium (Murashige & Skoog 1962), supplemented with 20 g/l sucrose and solidified with 0.7% agar (Britania®). The medium pH was adjusted to 5.7 with KOH (0.1 M and 0.5 M) and HCl (0.1 M and

0.5 M) and autoclaved for 17 min at 121 °C and 1,013 bar.

As preliminary assays for the *in vitro* propagation, nodal segments were tested with in the basal medium under different treatments: a) MS culture medium without plant growth regulators (control), b) MS culture medium supplemented with 1 mg/l of the synthetic cytokinin benzyl-aminopurine (BAP), and c) MS culture medium supplemented with 1 mg/l zeatin, as described by Gleddiei et al. (1985). Leaves were also tested with the same treatments (Gleddiei *et al*. 1985).

In both assays (*i.e*. nodal segments and leaves), 10 explants were used and two repetitions were performed. Each one was cultured in a 24 × 120 mm glass tube containing 10 ml of medium, covered with transparent film, and kept in a Sanyo culture chamber at 24 °C and under fluorescent tubes giving an irradiance of 52 mol × m^{-2} × s^{-1} and a 16 h photoperiod. The formation of an organogenic callus and the development of shoots were evaluated.

Based on the results obtained, the micropropagation of *S. sisymbriifolium* was carried out culturing the explants in the basal medium MS supplemented with 1 mg/l BAP. The culture conditions were the same as those described above. After 60 days of culture, the multiplication stage was considered finished.

For rooting, the shoots recovered from the two assays were transferred to the same medium but free of growth regulators. Once rooted, the plantlets were transferred for acclimatization in a greenhouse using plastic cups and a mixture of peat:perlite: vermiculite (3:1:1) (Guariniello *et al*. 2018). The nylon bags used to make the humidity chamber were gently perforated once a day until no inside condensation was detected. This feature determined the end of the acclimatization stage. After 30 days, plants were grown under standard greenhouse conditions.

Polyploidization assay

To establish a polyploidization protocol, based on the protocol of Iannicelli *et al*. (2016a), a 0.01% colchicine solution was added to the basal medium MS supplemented with 1 mg/l BA. This medium was autoclaved under the same previous conditions. Two replicates were performed with 10 *in vitro* nodal segments each, and the explants were cultured for 15 days. As a control treatment, 10 nodal segments were cultured on the same medium without colchicine. As colchicine is photosensitive, all treatments were maintained in darkness. After 15 days, all explants were transferred to the same medium but without colchicine, so they were also transferred to light.

The shoots were transferred every 30 days to the same fresh medium until the third subculture, in which the same medium did not contain BAP in order to start rooting. The rooted plantlets were rusticated with the same procedure mentioned above.

To determine the ploidy level, 20 samples corresponding to all the plants recovered from the colchicine treatment were analyzed with a flow cytometer (CyFlow Ploidy Analyser, Partec). The plants that resulted in autopolyploid were subsequently confirmed by chromosome counting. For the flow-cytometry measurements, and according to the manufacturer's instructions, approximately 0.5 cm^2 of leaf tissue taken from all the branches of the plant was submerged in a stripping buffer (Otto buffer I) consisting of 0.1M citric acid and 0.5% Tween 20 (Otto 1990) and chopped with a sharp razor blade. After filtration, the solution was stained with 4 µg/ml of a solution of 4',6-diamidino-phenylindole (DAPI) (Sigma D9542) in a buffer solution (Otto buffer II) composed of 0.4 M Na_2HPO_4 (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. To obtain the corresponding graphs, the data obtained by flow cytometry were run in the Flowing software 2.5.1. (University of Turku, Finland).

For chromosome counting, potted plant roots were collected and pre-treated with a 0.002% 8-hydroxyquinoline solution with 3.125 ppm cycloheximide (Cuco *et al*. 2003) for 4 h at 5 ºC, and then fixed in a 3:1 ethanol:acetic acid solution for 72 hours. Roots were hydrolyzed with 1N HCl for 10 min in an oven at 60 ºC, stained with Feulgen reagent for 90 min and treated with an enzymatic solution of 2% cellulases and 20% pectinases. Finally, the root tips were mounted with 2% acetic carmine and the preparations were observed under an Olympus BX 50 optical microscope and photographed with an Olympus DP72 digital camera using the CellSens Standard software.

The size of 10 young fully developed leaves (width and length) and 20 total expanded flowers from diploid and tetraploid plants was measured with a Mitutoyo calliper. The values obtained were analyzed by Excel, using the T Student test for data with different standard deviations.

The color of flowers and leaves was defined by means of the RHS Color Chart, to the eye of two people, in an environment well illuminated by natural light.

Results and Discussion

S. sisymbriifolium micropropagation

With an efficiency of around 25%, disinfection was the greatest obstacle to overcome in order to achieve an aseptic culture of *S. sisymbriifolium*. However, although few explants were recovered, it was possible to continue the assay and establish the response of the local genotype to the growth regulators, BAP and zeatin, proposed by Gleddiei *et al*. (1985).

After 15 days of culture, the nodal segments treated with these cytokinins showed the development of pre-existing buds, the incipient development of callus, and sectors of the explant with signs of browning. On the other hand, treated leaves also showed oxidation in some sectors and incipient callus development.

At 60 days of culture, after two subcultures, the number of shoots obtained from each treatment was counted. From the assay with leaves, through indirect regeneration, it was possible to recover 19 shoots from six explants only in the BAP treatment, which gives a multiplication rate of three shoots per explant. On the other hand, from the nodal segments treated with zeatin, it was possible to recover 12 shoots from the cultured explants, which gives a multiplication rate of 1.2 shoots per explant, whereas from those treated with BAP, it was possible to recover 23 shoots, which gives a multiplication rate of 2.3 shoots per explant.

Without losing sight of the preliminary condition of the assays of the present work, the results with the two cytokinins proposed by Gleddiei *et al*. (1985) (BAP and zeatin) for the regeneration of *S. sisymbriifolium* showed a comparable number for the treatment of nodal segments with zeatin and for that of leaves with BAP. In the treatment with 1 mg/l of BAP, an important difference was detected in the ability to generate outbreaks. Indeed, in the report by Gleddiei *et al*. (1985), this growth regulator showed a multiplication rate of 1 shoot per explant, while, in the present work, the rate observed was more than two-fold higher, after 60 days of culture, in both cases. It is difficult to find an adequate explanation for this difference, given that several aspects should be considered. The first could be the low reproducibility of *in vitro* plant tissue culture between laboratories. Also, considering the time elapsed between both studies (40 years), there could be differences in the quality of the reagents used. Regarding the mother plants, there may have been differences in the physiological state between both at the time of the corresponding tests. However, perhaps the most consistent guess could be the difference between genotypes. The fact is that the plants used in the present work could show higher sensitivity to BAP than those used by Gleddiei *et al*. (1985).

The different steps of *S. sisymbriifolium* micropropagation are shown in Figure 1: the initial explant (Fig. 1a), the explant with callus development and multisprouting (Fig. 1b), the rooted shoot (Fig. 1c), and the start of the acclimatization step (Fig. 1d). It is important to remark that, independently of their origin (pre-existing or *de novo*), the developed shoots were excised and transferred to the rooting medium (Fig. 1c). Only a few shoots showed signs of hyperhydricity.

The stages of rooting and rustication were accomplished satisfactorily with a rooting rate of 90%, and all the plants were transferred to the greenhouse, where they developed normally and were viable (Fig. 1e).

As can be observed in Figure 1, new shoots were regenerated from callus. It is known that genetic variability may occur in cultures when plant regeneration occurs by indirect organogenesis through a callus (Cardone *et al*. 2004). Moreover, Iannicelli *et al*. (2016b) found variations between the donor mother plant and plants derived from pre-existing meristems, suggesting that organized meristem cultures are not always genetically true-to-type. Therefore, it is not possible to be sure of the genetic fidelity of the regenerated plants until it is verified through the application of some type of molecular marker.

Polyploidization

From the results obtained in the preliminary *in vitro* establishment and propagation assay, the polyploidization experiment was designed using BAP as a growth regulator.

With an average of 2.3 shoots per explant after 60 days of culture, both in those treated with colchicine and in the control ones, the result was the same as that obtained in the *in vitro* introduction test. This would indicate that neither colchicine at the dose here applied nor the stage in the dark

Figure 1 – Progress of *Solanum sisymbriifolium* micropropagation – a) Nodal segment at the beginning of the culture. b) Aspect of one explant after 45 days of culture on MS medium supplemented with 1 mg/l BAP. The black arrows indicate the pre-existing shoots developed, whereas the red arrows indicate the *de novo* ones. c) Rooting stage. The arrows indicate the developed roots. d) Plantlet starts the acclimatization step. e) End of the micropropagation process with the plants under normal greenhouse conditions. Bar: 1 cm

affected the response of *S. sisymbriifolium* under the culture conditions proposed.

Of the total of 20 explants, with an average of 2.3 shoots per explant, five chimeras and two independent polyploidy events were obtained. However, only one was finally recovered as a viable individual.

Figure 2 shows the flow cytometry profiles for the solid tetraploid individuals, the five chimaeras and the control. It is a known fact that obtaining solid polyploidy is a stochastic phenomenon. It depends not only on the colchicine dose applied to the culture condition for the multiplication rate but also, according to our own experience, on having a clear regeneration timing of the culture. The knowledge of the right moment to expose the explants to colchicine and the morphogenic status of the culture to avoid chimeras and losses of material allow to achieve the highest number of solid tetraploid individuals (Iannicelli *et al*. 2016a; Escandón *et al*. 2007).

Table 1 shows differences in DNA amount and chromosome number. The chromosome count confirmed the data obtained by flow cytometry. Figure 3 shows the chromosomes of the wild genotype (Fig. 3a), with 2n = 24 chromosomes, and those corresponding to a duplicated individual (Fig. 3b), with 2n = 48 chromosomes.

Also, Table 1 describes the differences found between the diploid control plants and the recovered tetraploid. It is known that one of the traits that characterize tetraploids is the larger size of their organs (Petit & Callaway 2000).

In the present study, both leaves and flowers responded to this characteristic. In effect, in both cases, significant differences were observed in

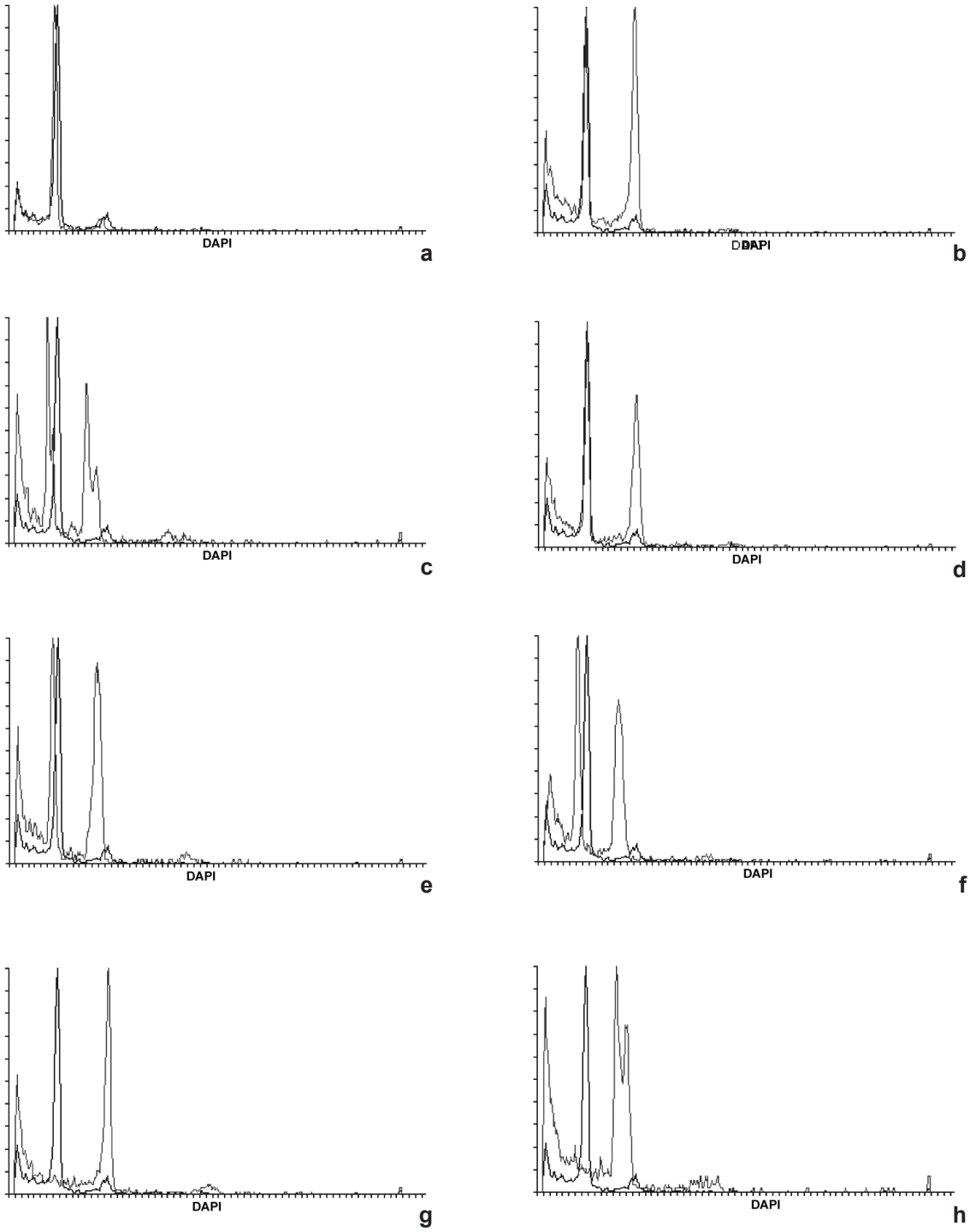

Figure 2 – Different profiles obtained via flow cytometry – a) Control + diploid plant. Control + b) Chimera 1. c) Chimera 2. d) Chimera 3. e) Chimera 4. f) Chimera 5. g) Tetraploid plant 9.1. h) Tetraploid plant 9.2. Control: in black line. Treated: in gray line. Abscissa axis: DNA content (arbitrary units). Ordinate axis: DAPI.

Table 1 – Summary of the differences in the size of leaves and flowers, DNA amount and chromosome number, between the original diploid individual and the recovered tetraploid. Different letters indicate significant differences (Student's test for different standard deviation, p>0.05).

Leaves	**diploid**	**tetraploid**
Length	10.99 ± 0.31[a]	13.30 ± 0.12[b]
Width	6.89 ± 0.42 [a]	10.04 ± 0.48[b]
Flowers		
Diameter	2.90 ± 0.02 [a]	3.91 ± 0.21[b]
Cytogenetic		
DNA content	8	16
Chromosome number	24	48

the size of these organs between the diploid and tetraploid individuals obtained. The size of the leaves of tetraploid individuals and the diameter of the flowers were significantly larger than those of diploid individuals. Figure 4 shows the difference in size between a fully extended young leaf of a diploid individual (Fig. 4a) and that of a tetraploid individual (Fig. 4b). Figure 5 shows the difference in size between a diploid flower (Fig. 5a) and a tetraploid flower (Fig. 5b).

The color measured in flowers and mature leaves by the RHS Color Chart ranged between cards 97D and 97C for flowers (white-violet). This variation would depend on the degree of maturity of the flower and not on the level of ploidy. For the leaves, the color ranged between cards 137A, 136A, 137A, and 139A for tetraploid leaves and matched card 131A for the diploid leaf. In the case of leaves, the RHS Color Chart indicates a greater intensity of bluish-green for the tetraploid relative to the diploid.

Figure 6 shows the differences in both the architecture and phenology between diploid and tetraploid *ex vitro* plants, which are noticeable, at least at this stage of development, in the length of the internodes and the time of flowering. These differences are characteristic of polyploid plants as, in general, polyploids show a compacter appearance and slower development than diploids,

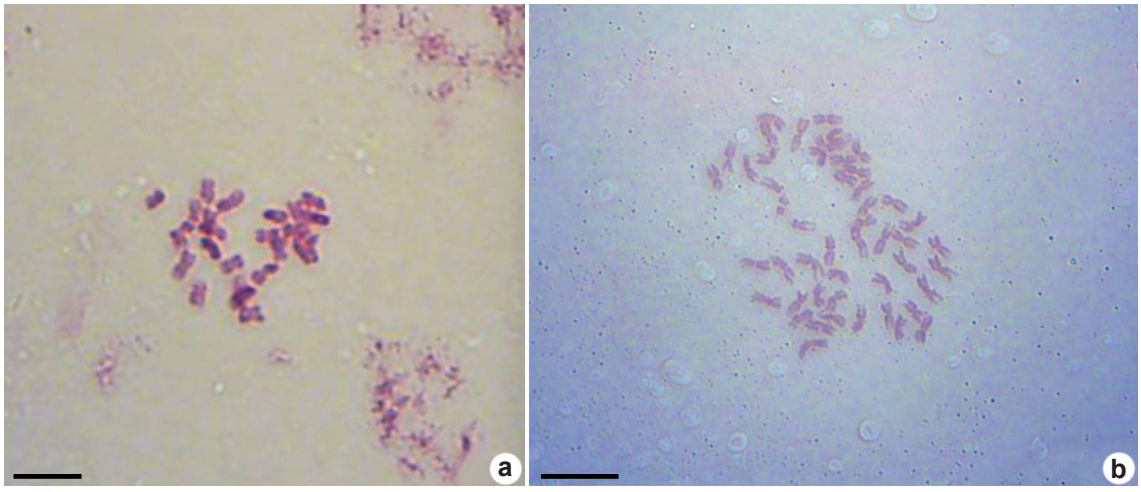

Figure 3 – Metaphase chromosomes of the diploid individual (panel "a") and the tetraploid individual (panel "b"). In panel "a", bar= 5 µm, in panel "b", bar= 10 µm.

Figure 4 – Size differences between a fully extended young leaf of a diploid individual ("a") and that of a tetraploid individual ("b").

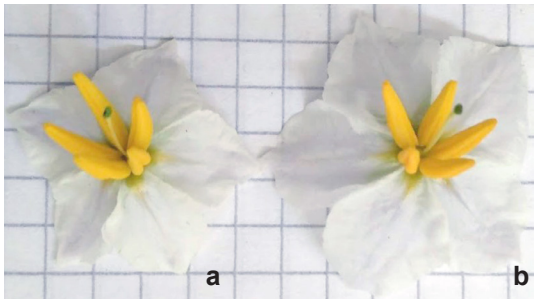

Figure 5 – Average flower size of the diploid individual (Panel "a") and the tetraploid individual (Panel "b").

as reported for *Lippia integrifolia* (Guariniello *et al*. 2020), *Cymbidium* Swartz (Zeng *et al*. 2020), a hybrid of the genus *Glandularia* (Gonzalez Roca *et al*. 2015), *Watsonia lepida* (Ascough *et al*. 2008), and *Mecardonia tennella* (Escandón *et al*. 2007), among others.

It is interesting to highlight that, regarding the variability observed and measured, such as the increase in the size of leaves and flowers, the retardation in growth that lead to a more compact appearance, and the changes in the intensity of colors, only the increase in the size of leaves and flowers and the retardation in growth could be attributed to the polyploid effect. The pigmentation of the flowers showed no significant changes other than those generated by the maturity of the organ. As for the difference in the intensity of the color of the leaves between the different levels of ploidy, it was very subtle, possibly due to some alteration in the regulation of the metabolic pathway and not, as expected, to the duplication of the gene dose, which could increase the intensity of the color. These differences in the polyploid effect would be in agreement with the non-linearity of the polyploidization phenomenon proposed (Iannicelli *et al*. 2020).

Obtaining synthetic autoploids has interesting advantages as a strategy applied to plant breeding. In fact, these new genotypes, whose genome, phenotype, physiology and metabolome are modified, show improved morphological,

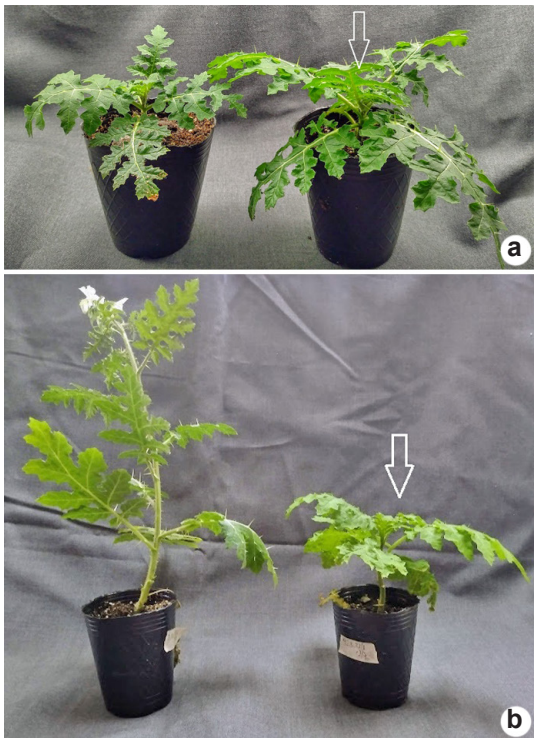

Figure 6 – Diploid and tetraploid plants at different stages of development. Panel "a": at an early stage. Panel "b": the same individuals three months later.

physiological and biochemical properties, as well as larger biomass (an effect pursued in forage production) and different colors of leaves and flowers (which generate new ornamental products) (Niazian & Nalousi 2020). These new genotypes also show an increase in the production of secondary metabolites, which provides them better resistance to stresses and may be profitable for the industry (Tossi *et al*. 2022; Sanjay 2021; Iannicelli *et al*. 2020; Niazian & Nalousi 2020).

However, to date, there is no general protocol to obtain artificial autoploids, and, since success is not guaranteed, it is important to consider the following premises: i) use a diploid species with a low number of chromosomes; ii) use an allogamous species; iii) use easy vegetative propagation (Cubero 2003). These characteristics will facilitate not only the obtaining of the new genotypes but also their subsequent handling. In this context, *S. sisymbriifolium* meets the aforementioned requirements and is also a very important species for the control of nematodes in horticulture (Perpétuo *et al*. 2021; Dandurand *et al.* 2019; Mitidieri *et al*. 2015; Pestana *et al*. 2014; Timmermans 2005), with interesting pharmacological potential due to the alkaloids present in its secondary metabolism (Ferro *et al*. 2005; Weissenberg 2001; Vieira & Carvalho 1993).

We were able to adjust a protocol for the micropropagation of *S. sisymbriifolium*. This is the first report on how to obtain tetraploids of *S. sisymbriifolium* via biotechnological tools. The new genotype could serve as input in a breeding program of this species to contribute to the control of soil pests in horticulture with less impact on the environment.

Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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