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Research article

Octoploid blueberry development for drought tolerance: A combined approach of *in vitro* polyploidization and somatic organogenesis ☆

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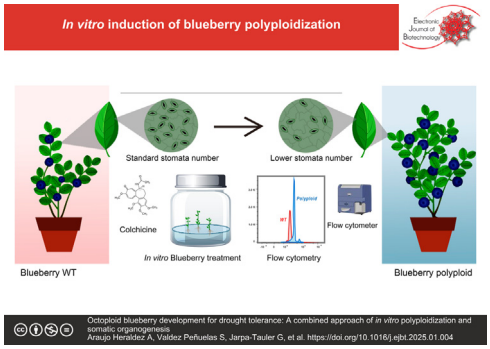
GRAPHICAL ABSTRACT

Octoploid blueberry development for drought tolerance: A combined approach of *in vitro* polyploidization and somatic organogenesis



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ABSTRACT

Background: The blueberry (*Vaccinium* spp.) is a fruit commercially known for its high quality and health benefits, particularly for its bioactive antioxidant compounds, which are important in the medical field. However, factors such as genotype, stage of fruit ripening and environmental conditions impact the biosynthesis of bioactive compounds in the berry, as well as their yield and cultivation costs. In Mexico, particularly in the state of Sinaloa, extreme climatic conditions limit the cultivation of blueberry and highlight the need for the development of new varieties with low chilling requirements and tolerance to drought conditions.

Results: Through the combined use of somatic organogenesis and *in vitro* polyploidization, genetic variability was promoted in the commercial blueberry plant variety "Biloxi". To achieve this purpose, blueberry microcuttings were treated with colchicine (0.02%) for six hours for 2, 4, 6 and 8 consecutive days and induced to form shoots *in vitro* with Zeatin (1 mg·L^{-1}). Out of 304 generated plants, 36 showed lower stomatal density and 9 lines showed higher stomatal density. Likewise, 5 and 49 lines presented lower and larger stomatal sizes, respectively. In 9 lines, a higher chlorophyll content was found (10% to

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Stomata
Vaccinium corymbosum L.

200%) compared to the control treatment. Ploidy analysis using flow cytometry showed the successful generation of four octoploid blueberry plants.
Conclusions: This work successfully generated new octoploid blueberry plants. Currently, all the lines that presented histological, biochemical and/or genetic modifications are being evaluated under greenhouse conditions for fruit quality and drought tolerance.
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1. Introduction

The blueberry is considered a berry native to North America, belonging to the genus *Vaccinium* which encompasses approximately 400 to 500 species [1]. Currently, the species with the greatest economic relevance are *V. corymbosum* L., *V. ashei Reade, V. angustifolium* and other *Vaccinium* species [2,3]. In Mexico, only varieties derived from *V. corymbosum* L. are cultivated. In recent years, these have experienced notable growth both in surface area and in volume of exports to North America, Asia and Europe. The extreme climatic conditions present in the State of Sinaloa limit the growth of this fruit tree and represent an opportunity to innovate in the development of new varieties adapted to these specific edaphoclimatic conditions.

Although the genetic improvement of blueberries began in the USA in the 1900′s with the outstanding contributions of Frederick Coville, numerous researchers have since contributed to the development of new varieties, focusing mainly on fruit quality, postharvest conditions and disease tolerance [4,5]. However, the requirements of basic chilling hours necessary for berry production initially restricted the cultivation of this species in warm climates. In the last decade, however, varieties with low chilling hour requirements have been developed, mainly through directed crossbreeding between Northern and Southern highbush, among others [4,6,7].

In the generation of these new varieties, biotechnological tools have been used with success, such as molecular markers and the induction of polyploidization *in vitro* [8,9]. These efforts have resulted in an increase in chromosome number, larger size of leaves, increased cell size, a greater number of chloroplasts per cell, and higher rates of photosynthetic enzymes and secondary metabolites observed [10]. Likewise, morphological and anatomical characteristics such as plant height, leaf area, stomatal size and density, and number of chloroplasts in guard cells are positively altered in polyploid blueberry plants by conferring greater tolerance to drought stress [11,12,13,14,15,16].

Recently, in Mexico, a Blueberry Improvement Program (BIP) focused mainly on developing varieties with lower chilling hour requirements and greater drought tolerance was initiated. To achieve this goal, a combined approach using polyploidization induction with the antimitotic agent colchicine and somatic organogenesis via *in vitro* culture was employed. This study reports the progress made in generating new octoploid blueberry lines with differences in number and stomatal size compared to control plants. It represents a first step in the search for blueberries with greater tolerance to drought in Mexico.

2. Materials and methods

2.1. Plant material and in vitro somatic organogenesis

The plant material consisted of stems from two-year-old southern highbush blueberry plants (*Vaccinium corymbosum* L.) of the commercial variety Biloxi. We selected this variety because it is very productive, has very low chill hour requirements for fruit production and is tolerant to high-temperature conditions such as those found in northern Mexico. Uniform segments of 10 to 15 cm in length were collected from plants growing in the greenhouse. The explants were first washed with commercial detergent and plenty of water to remove surface dirt. They were then disinfected in a laminar flow hood with a 0.18 mmol sodium hypochlorite (NaClO) solution containing a few drops of Tween20 for 40 min. Subsequently, they were immersed in 70% ethanol for 60 sec and finally, 3 to 5 washes were performed with sterile distilled water.

The explants were cultured for one month in flasks containing WPM (Woody Plant Medium) that included Zeatin (Zt) at a concentration of 0.5 $mg{\cdot}L^{-1}$. The flasks were incubated at a temperature of 23 ± 2°C, under a photoperiod of 16:8 h, provided by fluorescent lamps with a light intensity of 58 $\mu mol^{-2}sec^{-1}$. Sterile shoots were transferred to WPM multiplication medium containing Zt at a concentration of 1 $mg{\cdot}L^{-1}$ to allow shoot elongation. Subcultures were carried out every 30 to 40 d, according to the methodology described by Brenes Angulo et al. [1].

The induction of *in vitro* somatic organogenesis of blueberry was carried out using microcuttings measuring 1 to 2 cm in length, which were placed horizontally in contact with the same multiplication medium in Petri dishes. The pH of all culture media used was adjusted to 5.1 ± 1 and autoclaved at 121°C and 1.2 kg/cm^2 pressure (20 psi) for 15 min. The explants were incubated in a growth chamber under the conditions previously described.

2.2. In vitro polyploidization

For the induction of polyploidization, ten blueberry explants of approximately 1 to 2 cm in length (2 to 4 buds per explant) were placed horizontally in flasks for *in vitro* culture. These flasks contained multiplication solid medium modified with 0.02% colchicine, applied for six hours per day for 2, 4, 6 and 8 consecutive days. As a control treatment, microcuttings were used, maintained in WPM with Zt (1 $mg{\cdot}L^{-1}$), to induce the generation of de *novo* shoots [9]. The calculation of the percentage of sprouting in the polyploidization treatment was based on the total number of buds used per treatment as 100%. The total numbers of buds used were as follows: 324 for the control treatment, 342 for T1, 334 for T2, 318 for T3, and 317 for T4.

2.3. Histological and biochemical evaluation

The blueberry shoots generated under the different *in vitro* treatments, upon reaching 4 to 6 cm in height, were transferred to polypropylene 1 L cups containing substrate mix of peat and perlite (1:1). To induce rooting and subsequent *ex vitro* acclimatization, the bases of the shoots were treated with 0.1% indole butyric acid (IBA) for 30 min prior to transfer. The containers were maintained in a humidity chamber with a 16-h photoperiod at 58 $\mu mol^{-2}sec^{-1}$ and humidity of 95%. Acclimatization was per-

formed by gradually reducing the humidity over 3–4 weeks until it reached 85%, a level considered suitable for tissue culture-derived plantlet survival, following the methodology reported by Ghosh et al. [17]. Survival rate data for the plantlets were recorded six weeks after completing the hardening-off process.

The determination of density and stomatal size in the generated lines was carried out when the plants reached 20 to 30 cm in height. To do this, fully developed leaves located in the central part of the plants were used, obtaining an impression of the abaxial side of the leaves with the help of transparent adhesive tape. The impressions were placed on a slide for analysis under an optical microscope (SMZ800, Nikon Corp., Tokyo, Japan) following the methodology described by Romero-Romero et al. [18]. Stomatal density was determined at 10X magnification in four different fields of the middle part of the leaf, on a surface of 0.25 mm^2. Stomatal density was determined at 10X magnification in four different fields from the middle part of the leaf, covering a surface of 0.25 mm^2. Stomatal length was measured at 40X magnification, analyzing five stomata per field over a surface of 0.125 mm^2. The measurements were performed using Image J software (FIJI).

The quantification of total chlorophyll in plants was carried out using the method of Hiscox and Israelstam [19], using dimethyl sulfoxide (DMSO). To do this, discs measuring 1 cm^2 each (40 mg of total fresh weight) were collected and placed in a sterile glass tube, to which 7 mL of DMSO previously heated to 65°C was added. Subsequently, an incubation was carried out for 30 min at 65°C. Once the extraction was completed, the samples were removed and the glass tube was made up to exactly 10 mL by adding more DMSO. Next, 1 mL of each extract was transferred to glass cuvettes for analysis in a spectrophotometer (Thermo Scientific, BIOMATE 3S, EE. UU). The spectrophotometer was calibrated at zero absorbance using a pure DMSO blank. The absorbances of the blank and each sample were measured at 645 and 663 nm, respectively. The total chlorophyll concentration was calculated using the equations of Arnón [20]:

$$Chl\,a(gl^{-1}) = 0.0127A_{668} - 0.00269A_{645}$$

$$Chl\,b(gl^{-1}) = 0.0229A_{645} - 0.00468A_{668}$$

$$Chl\,tot(gl^{-1}) = 0.0202A_{645} - 0.00802A_{668}$$

2.4. Ploidy evaluation

Potentially polyploid plants were evaluated by quantifying DNA content using flow cytometry. For this, 20 mg of plant tissue was placed in the center of a Petri dish, to which 1 mL of Aru extraction buffer at 4°C was added. The tissue was finely cut with a scalpel. The mixture was then homogenized by pipetting, avoiding the formation of air bubbles and then the mixture was filtered through a 42 µm nylon mesh in a clean Petri dish. The recovered liquid volume was transferred to a new 1.5 mL tube that was previously labeled, and the stock solution of DNA-specific fluorochrome (DAPI 4 $\mu g \cdot mL^{-1}$) was added. The sample was subsequently incubated on ice at 4°C, until its analysis [21].

2.5. Statistical analysis

All experiments were carried out with three replicates per treatment, repeated three times. The results were subjected to a one-way analysis of variance (ANOVA) using a linear model. Statistical analysis of stomatal length, stomatal density, and chlorophyll was performed with one-way ANOVA. Multiple comparisons of the means were conducted using Tukey's test ($p \leq 0.05$). The Kruskal-Wallis test was used for data with non-parametric distribution, and Dunn's multiple comparison test ($p \leq 0.05$) was applied for comparisons of the means. Statistical analysis was performed using GraphPad Prism version 8.0.0 (for Windows, GraphPad Prism Software, San Diego, California USA).

3. Results and discussion

Polyploidy is one of the main pathways of speciation, enriching diversity in biological evolution. It has shown significant advantages in coping with limiting factors both biotic (viruses, fungi, bacteria) and abiotic (drought, salinity and extreme temperatures), particularly in the context of climate change [22]. In this study, the generation of blueberry plants with potential increased tolerance to abiotic stress, and thus improved adaptation to the extreme climatic conditions of certain regions in Mexico, was successfully achieved. The increase in DNA content in the Biloxi blueberry variety was demonstrated by flow cytometry, achieved through a combined *in vitro* treatment using blueberry microstakings and the antimitotic agent colchicine.

3.1. In vitro shoot regeneration and polyploidization by somatic organogenesis

Pathogen-free *in vitro* sprouts were successfully established from explants of cv. Biloxi, reaching a 91% sprouting rate, through the use of the phytohormone Zt (1 $mg \cdot L^{-1}$), which was statistically significant in comparison to the control treatment, which presented a sprouting of 43%. These results contrast with those obtained by Jimenez-Bonilla and Abdelnour-Esquivel [23] who in 2017 used the same growth regulator at concentrations of 0.5, 1 and 3 $mg \cdot L^{-1}$, obtaining sprouting percentages in *Vaccinium consanguineum* microcuttings of 7.1, 8.9 and 11.5% respectively. It is possible that these differences are related to the different genetics and regenerative capacity of *V. consanguineum* in relation to *V. corymbosum*. However, the high percentage of sprouting obtained in the Biloxy variety enables the planning of polyploidization induction.

After three subcultures of the obtained shoots, these were used for somatic organogenesis assays. The induction of direct organogenesis was observed within 4 to 5 d after the explants were transferred to the WPM multiplication medium. Initially, bud swelling was observed, followed by shoot elongation. The efficiency of *de novo* shoot induction in the medium added with Zt was 78%, while in the control treatment, that is, in the medium without the phytohormone, only 16.8% of shoot induction was generated. This result is in accordance with different reports in which the use of Zt favored the micropropagation of various blueberry varieties [24,25,26]. Following the establishment of the *in vitro* regeneration system from microcuttings of the Biloxi variety, polyploidy induction was carried out using microcuttings measuring 1 to 2 cm in length each containing 2 to 4 buds. In total, for the three repetitions of the test, 324, 342, 334, 318 and 317 buds were used for the control treatments, T1, T2, T3 and T4 respectively. The regenerative response obtained was evaluated at different exposure times (T1: 2 d, T2: 4 d, T3: 6 d and T4: 8 d) to the antimitotic agent colchicine at a concentration of 0.02%. The control treatment exhibited 60% sprouting, corresponding to 194 sprouts generated at 30 d posttreatment (dpt). In contrast, all treatments involving colchicine showed a proportional decrease in shoot regeneration as colchicine exposure and concentration increased (Fig. 1). Sprouting percentages of 47% (160 sprouts), 38.3% (128 sprouts), 32.4% (103 sprouts) and 28.3% (90 sprouts) respectively were observed in the different treatments T1, T2, T3 and T4. We attribute the superior results of the control treatment to the absence of colchicine in the culture medium. In blueberries and other plants, it

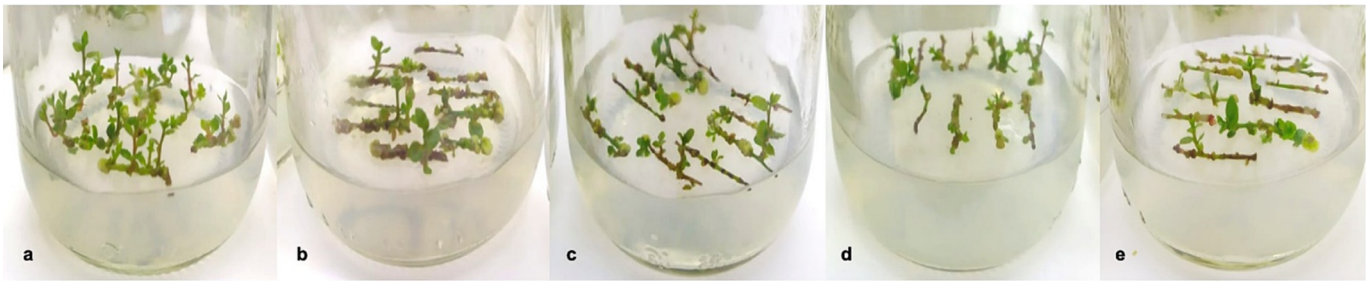

Fig. 1. Shoots generated *in vitro* from microcuttings of the Biloxi variety after 30 d of treatment with colchicine. (**a**) No-colchicine control, (**b**, **c**, **d** and **e**) Treatments T1, T2, T3 and T4 with 0.02% colchicine, for 6 h for 2, 4, 6 and 8 consecutive days respectively. The number of shoots/explant is presented as the mean ± standard deviation of three replicates, after counting the total number of sprouts per vial and dividing it by the total number of explants in the vial (ten explants per vial). Tukey's test ($p \leq 0.05$) performed multiple comparisons of the averages. Kruskal-Wallis's test was performed for samples with non-parametric distribution and multiple comparison of the averages was performed by Dunn's multiple comparison test ($p \leq 0.05$).

has been reported that *in vitro* polyploidization treatments reduce proliferative capacity in response to colchicine [15,27]. Our results are consistent with previous studies on blueberry by Dweikat and Lyrene [9], who reported that exposing explants of this species to 0.02% colchicine for 6 h per day over six days resulted in the successful production of hexaploid plants from triploid blueberry clones.

The favorable regeneration results obtained in the microcuttings of the Biloxi variety allowed us to advance in obtaining polyploid plants. In other species, polyploidization has been shown to enhance drought tolerance. For instance, a recent report on tetraploid citrus revealed slight decreases in photosynthetic parameters, such as Pn, gs, intercellular CO_2 concentration (Ci), Fv/Fm, and chlorophyll content, compared to diploids. However, tetraploids maintained high photosynthetic activity under water deficit conditions [28,29]. In this study, polyploidization was induced *in vitro* using colchicine as an antimitotic agent. By this procedure, a total of 481 blueberry shoots were obtained in response to the induction treatment. These were acclimatized *ex vitro* in pots, with a 95% establishment rate. Once the plants reached 10 to 20 cm in height, histological, biochemical, and genetic characterization was performed. From these shoots, 304 potentially polyploid plants and 10 plants from the control treatment were successfully generated under greenhouse conditions.

3.2. Evaluation of histological and biochemistry characteristics

Several reports have shown that polyploid plants show substantial improvements in cell size and structure, physiological and biochemical traits, gene expression and epigenetic modifications compared to their diploid counterparts [30]. Considering the high number of potential polyploids lines generated and recognizing that various investigations indicate that in the event of polyploidization, plants present changes at the stomatal level [13,31,32], we proceeded in a first stage of selection, to analyze the density and size of stomata in the newly obtained blueberry lines. As a result of this analysis, 36 lines showed a significant decrease in stomatal density, while 9 lines had a significant increase in the number of stomata, which corresponded to 11.8% and 2.9%, respectively, compared to the control treatment, as shown in Table 1 and Fig. 2a and Fig. 2b.

It is important to highlight that in the control line, an average of 80 to 85 stomata was observed in the analyzed area (0.25 mm^2) of the leaf tissue. In comparison, some of the newly generated blueberry lines showed between 52 and 60 stomata in the analyzed area, indicating a decrease in stomatal density of 27 and 37%. When analyzing stomata length (five stomata per field), the results demonstrated that 49 plants had larger stomata compared to the control. Likewise, in 5 regenerated plants, the stomata size was smaller than the control treatment (Table 1 and Fig. 2c and Fig. 2d). Interestingly, a correlation was observed where 5 plants with lower stomatal density simultaneously exhibited increased stomatal size.

These results agree to what was also reported by Ramírez et al. [11], where it was pointed out that in polyploid plants with nucleus having an increased number of chromosomes, their cells become longer and wider. Similarly, Manawadu et al. [33] reported that tetraploid radish plants presented larger stomata, but with a 30% decrease in their density compared to their diploid counterparts. Comparable findings were reported in blueberry by Marangelli et al. [15].

This histological characteristic, commonly observed in plants with increased ploidy, as demonstrated in this study, has not received sufficient attention and is often described merely as a consequence of polyploidization treatments. However, it has been recently reported that modifying the number and size of stomata simultaneously in a plant affects the Stomatal Pore Index (SPI) [34,35], altering gas exchange between the plant and the atmosphere. Notably, plants with a lower SPI have been shown to tolerate drought better by losing less water through transpiration without significantly affecting the photosynthetic process [34]. This suggests that these procedures could enhance Water Use Efficiency (WUE) in plants, a crucial trait for plants exposed to drought conditions caused by extreme environments or climate change impacts. Given the projections of increased drought in many countries of the planet, including Chile, Peru, and Mexico, this approach

Table 1
Characterization of stomatal density and size in potentially polyploid blueberry lines.

Treatment	Lower stomatal density*	Higher stomatal density*	Smaller stomata size*	Higher stomata size*	No differences	Total plants analyzed
T1	5	1	1	5	79	91
T2	17	4	1	18	47	87
T3	3	2	1	6	46	58
T4	11	2	2	20	33	68
Total	**36**	**9**	**5**	**49**	**205**	**304**

* Number of lines with significant differences in the indicated characteristic compared to the control plants.

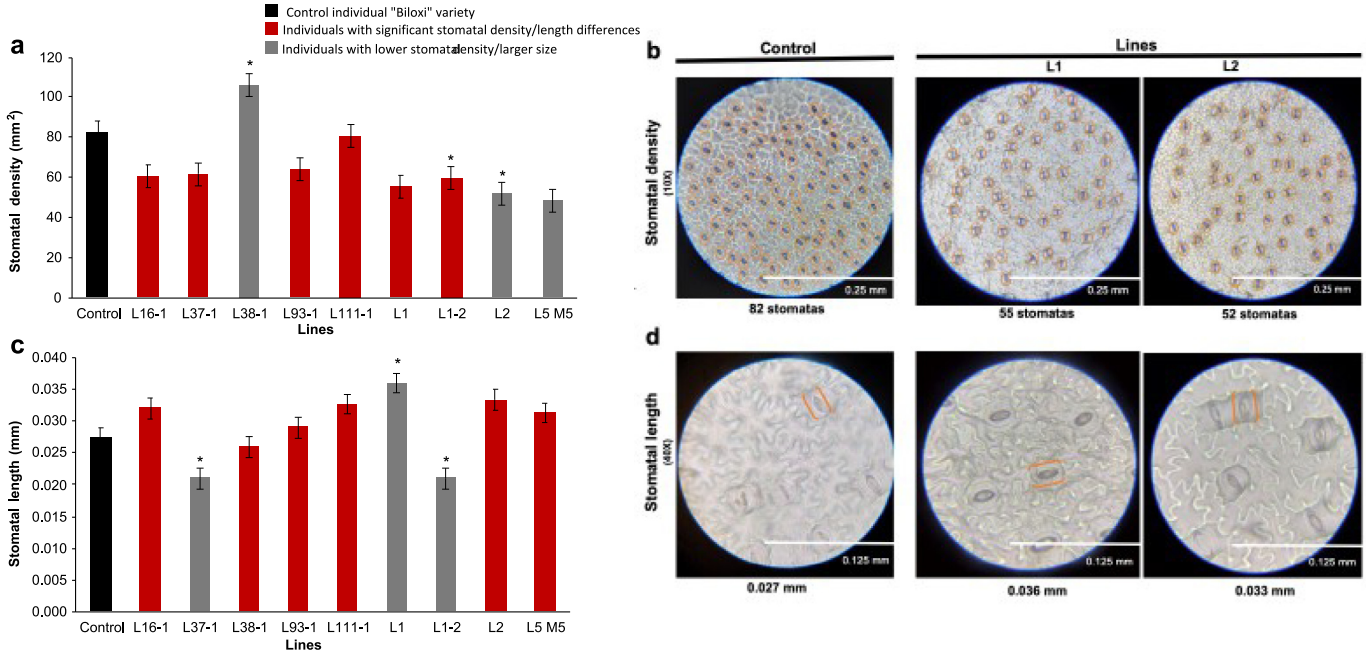

Fig. 2. Comparison in stomatal density and size between new potential polyploid blueberry lines and reference individual. (a) Number of stomata in mm^2, (b) Micrographs of stomatal density (10x), (c) Length of stomata in mm, (d) Micrographs of stomatal length (40x). Control individual "Biloxi" variety, Individuals with lower stomatal density or larger stomatal size, Individuals with significant diferences in stomatal density or stomatal length. Figures b and d show representative images of the density and size of stomata respectively in two potential polyploid lines and a control plant. Data are presented as mean ± standard deviation, and the asterisk denotes significant differences compared to the control ($p \leq 0.05$).

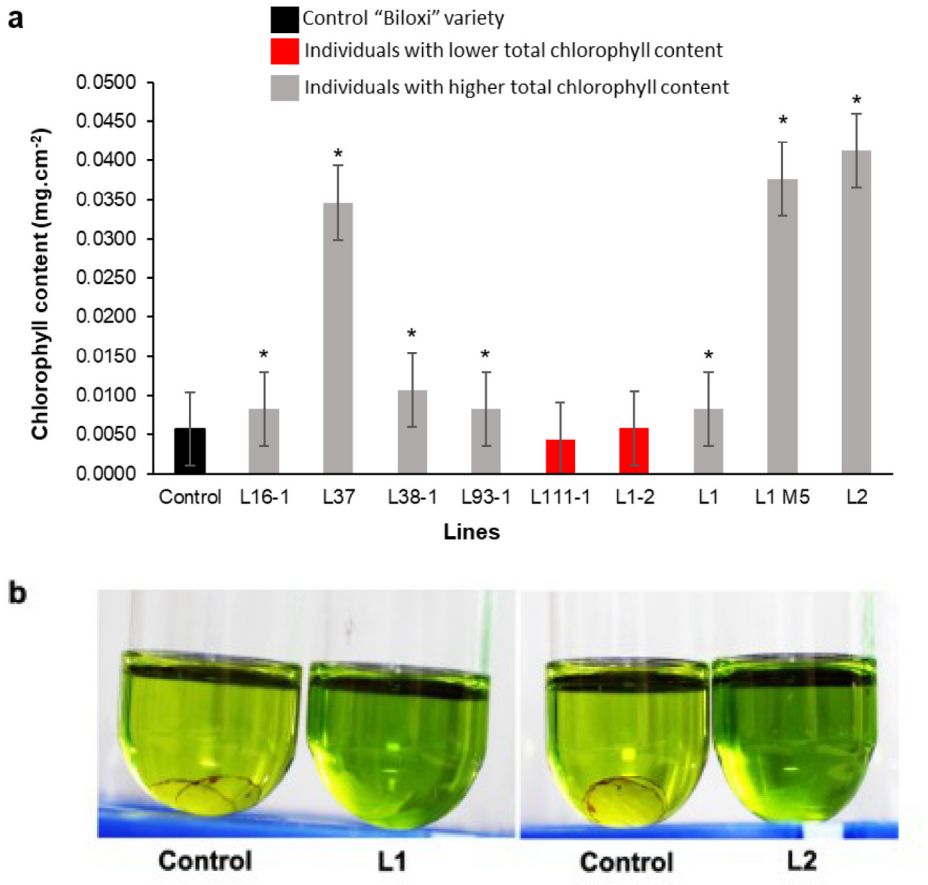

Fig. 3. Total chlorophyll content in the new potentially polyploid blueberry lines and the reference individual. (a) Control and potentially polyploid blueberry lines, (b) Chlorophyll extracts solution in control individual and potentially polyploid blueberry lines. Data are presented as mean ± standard deviation, asterisk denotes significant differences compared to the control ($p \leq 0.05$).

offers a powerful biotechnological tool to mitigate the adverse effects of climate change in a variety of agriculturally important plants. Given that the increase in plant ploidy correlates with an increase in the number of chloroplasts per cell, it was also decided to quantify the content of chlorophyll *a*, chlorophyll *b* and total chlorophyll content ($mg \cdot cm^{-2}$) following the methodology described by Hiscox and Israelstam [19], in all of the 99 blueberry plants generated that presented modifications related to stomatal density or size (Fig. 3a).

After determining the chlorophyll concentration, we observed that nine of the lines exhibited an increase in total chlorophyll content ranging from 10% to 200% compared to the control treatment. Among these, seven lines showed statistically significant differences. The extracts demonstrated varying shades and intensities of green, as illustrated in Fig. 3b. This increase in the concentration of chlorophyll in polyploid plants through this methodology suggests that some polyploid blueberry lines have possibly been generated, and that the quantification of chlorophyll by the described procedure can be used as an initial indicator for polyploidy evaluation in plants.

The results obtained in this work have been supported by studies that compared chlorophyll content with the identification and differentiation of polyploid and control acacia plants [36]. The variation in chlorophyll concentration between diploids and tetraploids of *Acacia mearnsii* supports the use of chlorophyll content as a marker of ploidy in young seedlings of this species [36]. Additionally, Warner and Edwards [10] concluded that as the number of chromosomes increases in *A. confertifolia* polyploid plants, DNA content, enzymatic activity, cell volume, and photosynthesis per cell also increased, validating the results obtained in this study.

3.3. Ploidy evaluation by DNA flow cytometry

After performing the histological analysis and determining the chlorophyll content of the potentially polyploid blueberry plants, their ploidy level was assessed. The plants were selected based on specific traits, prioritizing those with reduced stomatal density accompanied by an increase or decrease in stomatal size, as well as those that exhibited changes in chlorophyll content. A total of 32 blueberry plants were analyzed by flow cytometry, following the protocol described by Arumuganathan and Earle [21] and optimized by Lei et al. [37]. As a result of this analysis, it was verified that four of these plants showed an increase in their ploidy (Fig. 2d). It is important to note that polyploidization does not always generate advantageous traits for plants. Partial or global duplication of the genome produces redundant copies of genes and some of them accumulate mutations that can cause a gene to lose function and decrease the fitness of the plant [38]. Additionally, it has been reported that polyploidization in plants can be unstable and requires several cycles of sexual reproduction until the genome is stabilized [39]. In our work, it is relevant to highlight that the Biloxi variety is tetraploid [40] and therefore new poly-

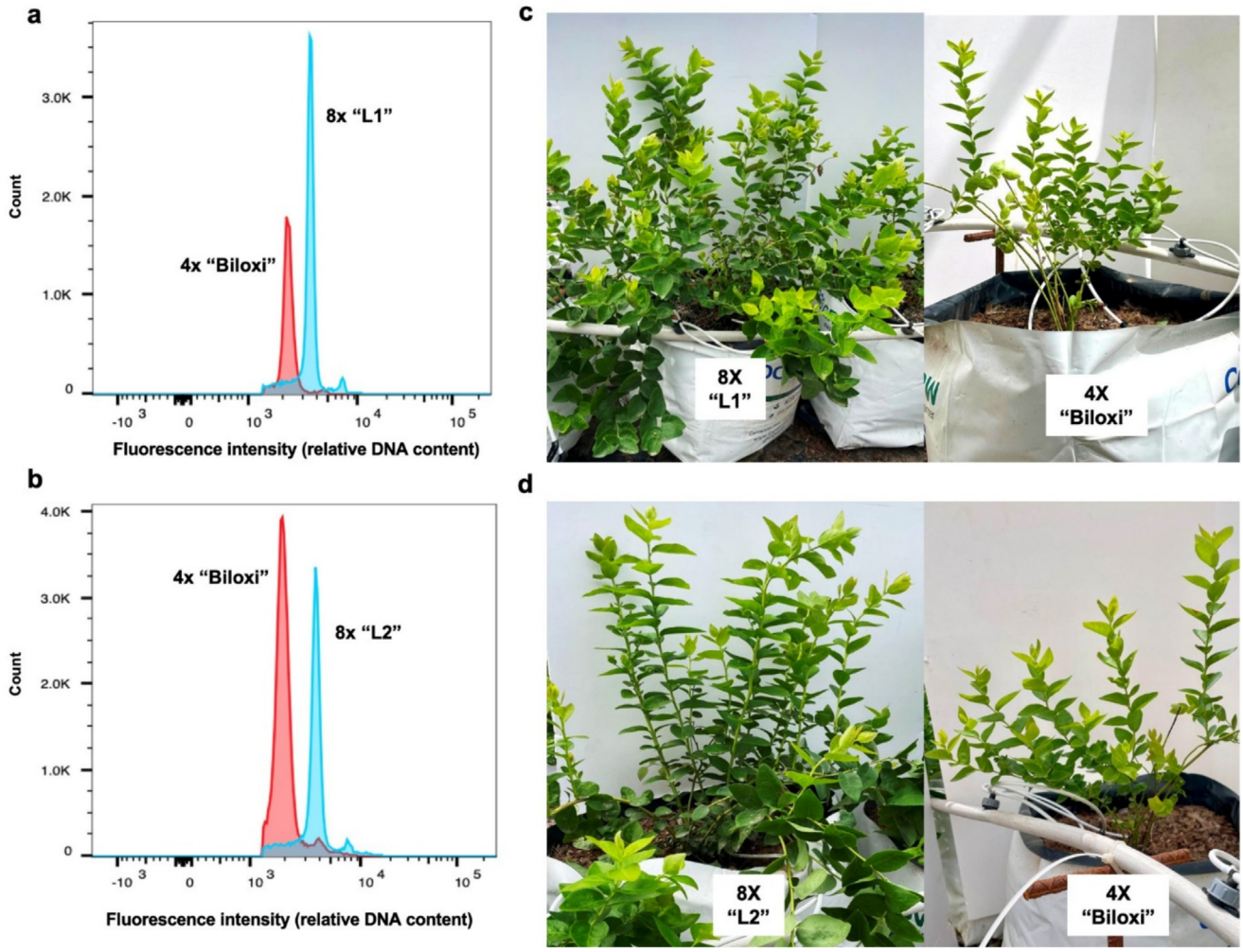

Fig. 4. Ploidy analysis by flow cytometry. (a and b) Histogram of the DNA content of the tetraploid control variety "Biloxi" and the generated octoploid Biloxy plants L1 and L2, (c and d) Greenhouse acclimated octoploid Biloxy plant "L1" and "L2" and control tetraploid "Biloxi" plants.

ploid blueberry plants that were generated are actually octoploid plants (Fig. 4a and Fig. 4b). These will reproduce asexually to preserve the characteristics of the generated plants, thereby reducing the possible genetic instability resulting from sexual reproduction. The promising results obtained with the induction of polyploidization in Biloxi explants, a variety that has very low chilling hour requirements and is also very tolerant to high temperatures such as those found in northern Mexico, are preliminary and thus we must carry out long-term studies. Therefore, complementary analyses of these plants in terms of their growth habit, physiological and productive behavior, as well as the characterization of their fruit and drought tolerance are ongoing efforts. We hope to better document through these studies the real contribution of this strategy as a genetic improvement tool, which could increase drought tolerance in blueberries and other species of interest. This method of inducing polyploidization for this trait, if successful, would allow the development of commercial blueberry plants with greater drought tolerance, and could potentially be applied to other species of agricultural interest in other areas of the planet affected by drought.

4. Conclusions

This work shows successful results in the combined use of *in vitro* polyploidization and somatic organogenesis induction treatments to obtain octoploid blueberry plants with differences in size and stomatal density. This is a first step to obtain new blueberry lines with the potential to be more tolerant to drought, offering a promising biotechnological solution for the adaptation of blueberries to extreme climatic conditions. Experiments evaluating physiological parameters of drought tolerance in these plants should be carried out.

CRediT authorship contribution statement

Alejandra Araujo Heraldez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Susana Valdez Peñuelas:** Writing – review & editing, Writing – original draft, Formal analysis. **Gabriela Jarpa-Tauler:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Aparna Banerjee:** Writing – review & editing, Writing – original draft. **Kattia Núñez-Montero:** Writing – review & editing, Writing – original draft, Visualization. **Patricio Arce-Johnson:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Jesús L. Romero-Romero:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Supplementary material

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Data availability

Data will be made available on request.

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